

IMMUNOLOGICAL STUDIES IN DIABETES MELLITUS

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### DECLARATION

I hereby declare that this thesis has been composed by myself and that the work presented therein is my own. In the case of studies involving collaboration with other workers, the contribution of these workers is acknowledged individually in the relevant sections of the thesis and in the published papers arising therefrom.

Angus C. MacCuish.



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The/

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A.C. MacCuish, E.W. Barnes, W.J. Irvine and L.J.P. Duncan.

Published in The Lancet, ii, 1529-1531 (1974).

- II/2 Autoimmune diabetes mellitus.

A.C. MacCuish.

Published in The Lancet (Leading Article), ii, 1549-1551 (1974).

- II/3 Cell-mediated immunity to human pancreas in diabetes mellitus.

A.C. MacCuish, Jennifer Jordan, C.J. Campbell, L.J.P. Duncan and W.J. Irvine.

Published in Diabetes, 23, 693-697 (1974).

- II/4 Cell-mediated immunity in diabetes mellitus: lymphocyte transformation by insulin and insulin fragments in insulin-treated and newly-diagnosed diabetics.

A.C. MacCuish, Jennifer Jordan, C.J. Campbell, L.J.P. Duncan and W.J. Irvine.

Published in Diabetes, 24, 36-43 (1975).

- II/5 A rapid micromethod for the phytohaemagglutinin-induced human lymphocyte transformation test.

W.J. Penhale, Anne Farmer, A.C. MacCuish and W.J. Irvine.

Published in Clinical and Experimental Immunology, 18, 1550167 (1974).

II/6 Phytohaemagglutinin transformation and circulating lymphocyte subpopulations in insulin-dependent diabetic patients.

A.C. MacCuish, S.J. Urbaniak, C.J. Campbell, L.J.P. Duncan and W.J. Irvine.

Published in Diabetes, 23, 708-712 (1974).

II/7 Autoimmunological aspects of diabetes mellitus.

A.C. MacCuish and W.J. Irvine.

Published in Clinics in Endocrinology and Metabolism, 4, 435-471 (1975).

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### ABSTRACT

Clinical and serological associations of insulin-dependent (Type 1) diabetes mellitus (IDDM) with the classical organ-specific autoimmune diseases are reviewed. The significance of the insulinitis lesion for the concept of autoimmune diabetes mellitus in man and in experimental animal models is discussed. The results of the following original in vitro studies of human diabetes mellitus are presented:

1. Humoral autoimmunity was studied with the indirect immunofluorescence technique, using fresh human pancreas as tissue substrate. An autoantibody directed against the endocrine cells of the islets of Langerhans was detected in sera from 51 of 353 diabetics tested. All but 3 antibody-positive patients had insulin-dependent diabetes. Islet-cell antibody prevalence was 30% in a group of 46 diabetics with coexistent overt autoimmune disease; 18% in a group of 107 diabetics with circulating thyrogastic autoantibodies; and 9% in a group of 200 diabetics with no clinical or serological evidence of autoimmune disease. The antibody was found in only 2 of 200 non-diabetics with thyrogastic or adrenal autoimmunity (one of whom subsequently developed IDDM) and in none of 350 normal subjects. Islet-cell antibody was shown to be a complement-fixing immunoglobulin of IgG class while the antigen against which it is directed (common to all cell types in the pancreatic islets) is probably microsomal in nature.

Subsequent extensive studies which have examined the significance of this pancreatic islet-cell antibody, now termed pancreatic cytoplasmic islet-cell antibody (ICA), are reviewed, with particular reference/

reference to its relationship to the duration of clinical IDDM, its prevalence in IDDM with coexistent autoimmune disease, and its value as a marker for the eventual development of IDDM in patients with non-insulin-dependent (Type 2) diabetes (NIDDM) and in nondiabetic subjects. The detection and possible significance of other antipancreatic autoantibodies is briefly described.

2a. Cell-mediated autoimmune mechanisms were investigated with the leucocyte migration inhibition test (LMT) and antigen-induced lymphocyte transformation tests. Using the LMT, cellular hypersensitivity was found to antigen of human pancreas homogenate in 29 of 101 diabetics and to an antigen of human islet-cell tumour (insulinoma) in 14 of 28 diabetics. The difference in response between diabetics and controls was confined to insulin-dependent patients, whether untreated (newly diagnosed) or insulin-treated at the time of study; there was no distinction between normal subjects and non-insulin-dependent diabetics treated by diet or oral hypoglycaemics. The use of liver mitochondria, insulin and glucagon as antigens in the LMT did not induce inhibition of leucocyte migration in diabetics or controls.

When considered in conjunction with other studies, these results suggest that cell-mediated autoimmunity to antigens which are organ-specific (pancreatic) and species-non-specific is demonstrable in a proportion of insulin-dependent diabetics, including newly diagnosed patients who have never received insulin.

2b. The ability of bovine and porcine insulin antigens to induce lymphocyte transformation was tested with cells from 30 normal controls, 50 established insulin-dependent diabetics with no clinical/

clinical evidence of insulin allergy, and 15 newly-diagnosed diabetics. Lymphocytes from 27 diabetics showed significant blastogenesis to insulin, as compared to 2 controls: the phenomenon was found in both established and newly-diagnosed patients, including 4 who had never taken insulin. The separate chains of insulin were further used to demonstrate that isolated B chain was a potent stimulator of blastogenesis but that isolated A chain was without significant effect.

These results indicate that cellular hypersensitivity to insulin is relatively common in insulin-treated patients without in vivo evidence of allergy (i.e. no immediate or delayed type cutaneous reaction to injected insulin, daily insulin requirement of less than 200 units); they further suggest that true autoimmunity to insulin may also be present in untreated diabetics. The insulin chain experiments might suggest that B chain is the major antigenic site determining cellular hypersensitivity to insulin: alternatively the negative response to A chain might merely reflect the damage sustained by this chain during the splitting of insulin.

3. The correlation of humoral and cellular autoimmunity was assessed by testing insulin-dependent diabetics (including newly-diagnosed patients), with and without circulating ICA, for their response to the LMT using an antigen of human islet-cell tumour (insulinoma). Migration inhibition was found in 50% of antibody-positive diabetics and in an equal number of antibody-negative patients. The significance of the weak correlation between humoral and cellular anti-pancreatic autoimmunity in these patients is discussed.

4. General immune function was assessed by mitogen-induced lymphocyte/

lymphocyte transformation tests and measurement of circulating lymphocyte subpopulations. The lymphocyte transformation response to phytohaemagglutinin (PHA) was found to be identical in a group of 40 well-controlled insulin-dependent diabetics and matched normal subjects: however transformation was grossly depressed in 14 poorly-controlled insulin-dependent diabetics. Subpopulations of T and B lymphocytes were measured in the same subjects: the results were identical in normals, well-controlled and poorly-controlled diabetics, each group showing an average of 63% T and 20% B cells in peripheral blood. The depressed PHA response in poorly-controlled diabetes seemed to reflect transient metabolic disturbance, rather than inherent immunologic abnormality, and returned to normal as the metabolic abnormalities were corrected. Serum samples from poorly-controlled diabetics were further shown to inhibit the PHA-induced transformation of lymphocytes from normal subjects, while serum removed from the same patients after correction of their metabolic abnormalities had no such inhibitory effects. Additional experiments indicated that hyperglycaemia and high levels of free (unsaturated) fatty acids both contributed to the inhibitory effects of sera from poorly-controlled diabetics.

These studies suggest that metabolic decompensation in diabetes is causally associated with depression of cell-mediated immune function, the depression being reversed by correction of the metabolic disturbance.

5. Immune abnormality in relation to the complications of diabetes was examined by a serological study of the prevalence of antibodies to non-pancreatic antigens in 400 diabetics and matched normal controls/



controls. Antibodies to thyroid cell and gastric parietal-cell occurred more than twice as commonly in diabetics as in non-diabetics, the excess frequency being found almost entirely in insulin-dependent patients. Thyrogastic antibody prevalence in insulin-dependent diabetes was not influenced by the age of onset or duration of disease, nor by the presence or absence of clinical microangiopathy.

All the above studies have indicated that insulin-dependent diabetics are immunologically distinct from non-insulin-dependent patients. Antipancreatic humoral and cellular autoimmune phenomena are both demonstrable in a proportion of insulin-dependent diabetics and autoimmunity may be of aetiological importance in this type of diabetes mellitus. The possible significance of autoimmunity in the aetiology of Type 1 diabetes, with particular reference to the role of viral infections and genetic factors, is briefly considered.

## CHAPTER ONE

### INTRODUCTION: CLINICAL AND SEROLOGICAL ASSOCIATIONS OF DIABETES MELLITUS WITH ORGAN-SPECIFIC AUTOIMMUNITY: THE INSULITIS LESION IN MAN AND EXPERIMENTAL ANIMAL MODELS OF IMMUNE DIABETES MELLITUS.

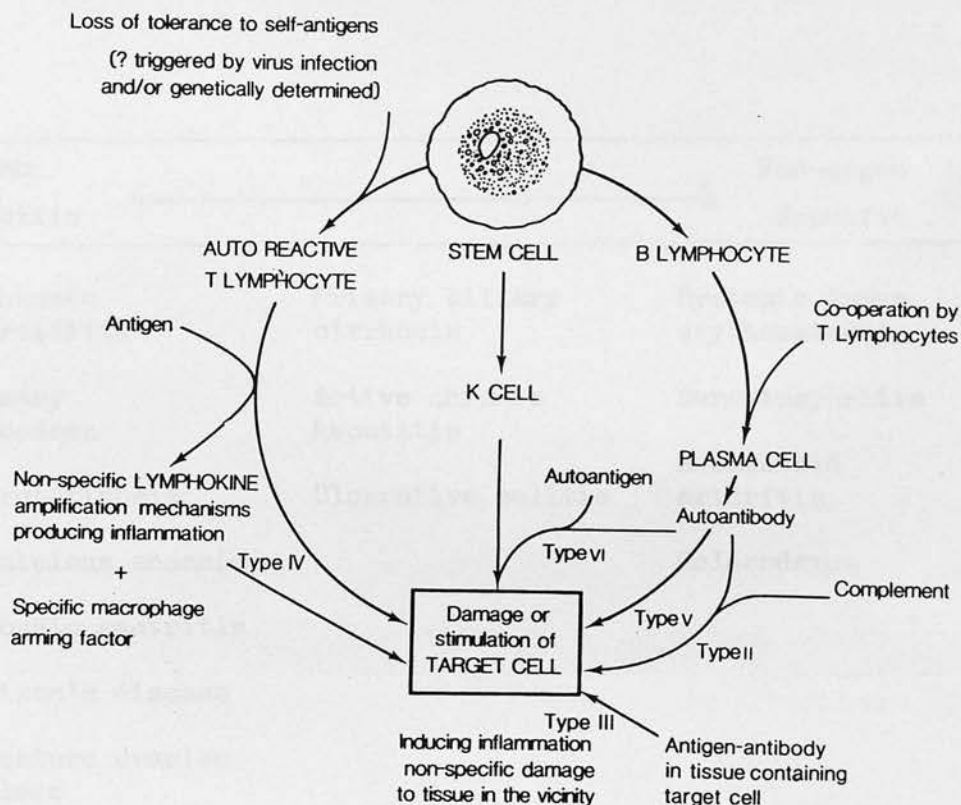
#### 1/1 The concept of autoimmunity

An autoimmune disease may be defined as a condition in which 'self' components (that is, components of the body's own tissues) are recognised as antigens by the lymphoid system of the affected individual. The consequence of such recognition is the appearance of immunologically competent cells, and the production of auto-antibodies, directed against body tissues. Implicit in this definition is the assumption that sensitised cells and autoantibodies have a pathogenic role in the development of the disease, as opposed to the circumstances in which transient and ostensibly harmless antibodies appear as the sequel to tissue damage. An example of the latter phenomenon would be the formation of heart antibodies after myocardial infarction. The role of autoimmunity in many disorders is often not sufficiently clearcut to permit the above assumption; nonetheless in the present state of knowledge, it is acceptable to apply certain criteria that allow a disease to be considered as being of autoimmune aetiology. These criteria are summarised in table 1/1, while a broad outline of the interactions of various immune mechanisms implicated in the production of autoimmune disease is given in figure 1/1.

As Roitt (1980) has indicated, autoimmune disorders are conventionally regarded as forming a spectrum of disease (table 1/2). At one/

TABLE 1/1Criteria for a disease of autoimmune aetiology

1. Presence of circulating autoantibodies in the serum (humoral autoimmunity).
2. Presence of circulating lymphoid cells sensitised to tissue autoantigens (cell-mediated autoimmunity).
3. Identification of the autoantigen(s).
4. Presence of tissue lesion(s) with lymphocytic or plasma cell infiltration.
5. Aggregation of the disease in the relatives of affected persons.
6. Aggregation of other autoimmune phenomena (subclinical or overt) in affected persons and/or their families.
7. Reproduction of similar disease in experimental animals by autoimmunisation techniques.

FIGURE 1/1

The interactions of immune responses Types II-VI in the production of autoimmune disease. Cell-mediated immune mechanisms lie broadly on the left of the figure, humoral mechanisms on the right; but co-operation between T and B lymphocytes is also displayed.

From Irvine (1979)

TABLE 1/2The spectrum of autoimmune disease

Organ Specific	←————→		Non-organ Specific
Hashimoto thyroiditis	Primary biliary cirrhosis		Systemic lupus erythematosus
Primary myxoedema	Active chronic hepatitis		Dermatomyositis
Thyrotoxicosis	Ulcerative colitis		Rheumatoid arthritis
Pernicious anaemia			Scleroderma
Atrophic gastritis			
Addison's disease			
Premature ovarian failure			
Idiopathic hypoparathyroidism			

Modified from Roitt (1980)



one end of the spectrum are the organ-specific diseases, characterised by infiltration of the affected organ with mononuclear cells, destruction of the organ's normal architecture and the appearance of circulating serum antibodies directed specifically against constituents of the affected organ. It will be noted from table 1/2 that hormone-producing organs seem to be peculiarly vulnerable to involvement by this type of autoimmune disease process; examples of endocrine autoimmunity include not only Hashimoto thyroiditis and primary hypothyroidism (Doniach and Roitt, 1974) but also thyrotoxicosis (Adams et al, 1974; Kendall-Taylor, 1975), pernicious anaemia and atrophic gastritis (Irvine, 1965; Roitt et al, 1965), idiopathic Addison's disease (Blizzard and Kyle, 1963; Irvine and Barnes, 1972, 1975), premature ovarian failure (Irvine and Barnes, 1974, 1975) and idiopathic hypoparathyroidism (Blizzard et al, 1966; Irvine and Scarth, 1969). The centre of the spectrum contains those disorders where the infiltrative lesion tends to be localised to a single organ but the autoantibodies are non-organ specific. Primary biliary cirrhosis is a typical example of 'mid-spectrum' autoimmune disease; the small bile ductule is the main target for mononuclear infiltration but the serum antibodies, mainly to mitochondria, are not specific for the liver (Doniach et al, 1966). The other end of the spectrum consists of autoimmune diseases which are clearly non-organ specific, and systemic lupus erythematosus is an example of a condition in which both lesions and autoantibodies are not confined to a single organ.

#### 1/2 The criteria for autoimmune diabetes mellitus

Diabetes mellitus has been defined as 'a state of chronic hyperglycaemia/

hyperglycaemia which may result from many environmental and genetic factors, often acting jointly' (World Health Organisation, 1980). The hyperglycaemia may be due to a lack of insulin or to an excess of factors that oppose its action. It is now recognised unequivocally that there are two main and distinct types of primary diabetes which are presently classified by reference to therapy required: Type 1 diabetes, otherwise termed insulin-dependent diabetes (IDDM) and Type 2 diabetes, otherwise termed non-insulin-dependent diabetes (NIDDM). Type 1 diabetics are ketosis-prone individuals who are absolutely dependent on daily injections of exogenous insulin for preservation of life; this is the common form of diabetes presenting in individuals under the age of 30 years but is not infrequent in elderly patients. Type 2 diabetics have a milder syndrome in which hyperglycaemia can be contained by dietary carbohydrate restriction or oral hypoglycaemic drugs; these patients are not prone to ketosis, are usually middle-aged or elderly and are frequently obese. The clinical classification of diabetes mellitus has undergone several major revisions in the past twenty years, and the terms 'juvenile-onset diabetes' for Type 1 patients and 'maturity-onset diabetes' for Type 2 patients have largely been abandoned. The modern classification of diabetes was exhaustively considered at an International Workshop organised by the National Diabetes Data Group of the National Institutes of Health, Bethesda, U.S.A., which met between 1978 and 1979. Its recommendations (NDDG, 1979) were subsequently adopted as an interim classification by the WHO Expert Committee on Diabetes (WHO, 1980), are summarised in table 1/3 and are now coming into general clinical usage. In proposing this new classification/

TABLE 1/3Diabetes Mellitus

- Type 1            -    Insulin-dependent type (IDDM)
- Type 2            -    Non-insulin-dependent type (NIDDM)
  - (a) Non-obese NIDDM
  - (b) Obese NIDDM
- Other Types    -    Including diabetes mellitus associated with certain conditions and syndromes:
  - (1) pancreatic disease, (2) disease of hormonal aetiology, (3) drug- or chemical-induced condition, (4) insulin receptor abnormalities, (5) certain genetic syndromes, (6) miscellaneous.

The clinical classification of diabetes mellitus (DM).  
 Simplified from the classification scheme prepared by the National Diabetes Data Group of the National Institutes of Health, USA (1979) and adopted by the Second Report of the WHO Expert Committee on Diabetes Mellitus (1980).



classification, the NDDG took special cognisance of a large corpus of information which had become available regarding the aetiology of diabetes, in particular of insulin-dependent diabetes, which it summarised thus: 'Evidence regarding etiology (of Type 1 diabetes) suggests genetic and environmental or acquired factors, association with certain HLA types, and abnormal immune responses, including autoimmune reactions' (NDDG, 1979). The purpose of this thesis is to review some of the evidence for immunologic abnormalities in diabetes mellitus and in particular to describe some original studies designed to examine the hypothesis that some cases of human diabetes mellitus may represent a true organ-specific autoimmune disease.

By analogy with the known immunopathology of endocrinopathies in which autoimmunity is established or postulated as a major aetiological factor, it is possible to apply certain criteria which must be met before the concept of an autoimmune form of diabetes could be considered viable: these criteria are summarised in table 1/4.

First, it is necessary to identify a close clinical connection between diabetes and established autoimmune disease. The autoimmune endocrinopathies are known to be intimately inter-related, they may coexist in the same patient, and they may appear in a familial pattern. Such diseases should therefore be over-represented, in this fashion, in a diabetic population.

Second, a significant association of diabetes with subclinical or preclinical autoimmunity should also exist, i.e. sera from diabetics should contain a high prevalence of organ-specific auto-antibodies directed against the thyroid, the stomach and the adrenal cortex. Multiple serum antibodies of this nature are again a constant/

TABLE 1/4The criteria for 'autoimmune diabetes mellitus'

1. Close clinical association with autoimmune endocrinopathies.
2. High prevalence of serum autoantibodies to nonpancreatic antigens.
3. Mononuclear cellular infiltration of the endocrine pancreas.
4. Presence of circulating lymphoid cells sensitised to antigens of the endocrine pancreas.
5. Presence of serum autoantibody directed against the endocrine pancreas.
6. Induction of diabetes by autoimmune mechanisms in experimental animals.

constant feature of accepted autoimmune disease and are independent of the increasing antibody prevalence with advancing age in the normal population. These first two criteria would provide good indirect evidence for an association of diabetes with autoimmunity.

More direct evidence of an immune lesion in diabetes might first be sought by examining the pathology of the endocrine pancreas at the onset of clinical diabetes, looking in particular for the mononuclear cell infiltrate of lymphocytes or plasma cells that is an established feature of thyroid, gastric and adrenal autoimmunity. It would further be necessary to demonstrate in diabetics that the serum contained an autoantibody directed against the endocrine pancreas and that circulating lymphoid cells were sensitised to antigens derived from the substance or hormones of the endocrine pancreas.

Finally, it would be equally important to demonstrate that clinical carbohydrate intolerance in experimental animals could be induced by immunological mechanisms, and that such intolerance was accompanied by appropriate evidence of an autoimmune lesion.

It is now proposed to examine the existing evidence for autoimmunity in diabetes using each of these criteria in turn. This chapter considers the clinical and serological associations of diabetes with autoimmune disease, the pathology of the endocrine pancreas in diabetes and the animal models of experimental immune diabetes. Subsequent chapters will review the evidence for humoral and cell-mediated autoimmunity in diabetes in the context of original studies undertaken by the author.

### 1/3 The clinical associations of diabetes with autoimmune disease

Some of the earliest and most compelling indirect evidence for abnormal/

abnormal immune mechanisms in diabetes has been provided by the clinical observation of an association between diabetes and the classical organ-specific autoimmune endocrinopathies. Coexisting diabetes and adrenal insufficiency in a single patient was first recorded in the mid-nineteenth century (Ogle, 1866), barely eleven years after Addison's classical description of the disease that bears his name; and numerous subsequent studies have reinforced the view that the two disorders - one common, one rare - are linked more intimately than can be accounted for by chance (Balfour and Sprague, 1949; Faber and Gronbaek, 1956; Beaven et al, 1959; Solomon et al, 1965; Irvine and Barnes, 1972; Nerup and Binder, 1973; Nerup, 1974; Irvine and Barnes, 1975; Irvine et al, 1980a; Nerup et al, 1980). From these and other studies the prevalence of diabetes in Addison's disease has been reported as between 7 and 23 per cent, with an approximate average of 18 per cent (Irvine and Barnes, 1975), thus representing an excess of at least ten-fold over the estimated prevalence (about 1.5 per cent) of diabetes in the overall population of Britain or the United States (Marks et al, 1971). Fewer investigators have attempted to estimate the prevalence of Addison's disease in diabetes but Kozak (1971) recorded a figure of 0.032 per cent among forty-three thousand newly-diagnosed cases of diabetes at the Joslin Clinic and Nerup (1974) reported a very similar figure (0.028 per cent) when he examined the prevalence of Addison's disease in a population of fifty thousand Danish diabetics. The prevalence of Addison's disease in the general population has been estimated at between 0.0039 per cent (Stuart-Mason et al, 1978) and 0.0060 per cent (Nerup, 1974); thus the excess prevalence of Addison's/



Addison's disease in diabetics appears to be in the order of five-fold over the general population. The development of more sophisticated diagnostic aids, in particular the ability to detect organ-specific adrenal antibodies (Anderson et al, 1957; Blizzard and Kyle, 1963), has confirmed that diabetes is usually associated with idiopathic (autoimmune) adrenal failure rather than tuberculous adrenal destruction, and this is borne out by pathological studies. One extensive review of the literature noted idiopathic adrenal atrophy in 74 per cent, and tuberculous adrenal involvement in only 22 per cent of those patients with dual disease who came to autopsy, and a similar pattern of adrenal pathology was demonstrated in diabetics with combined thyroid and adrenal dysfunction (Solomon et al, 1965). The combination of thyroid and adrenal insufficiency, which is sometimes referred to as Schmidt's syndrome (Schmidt, 1926), is a particularly striking expression of a strong autoimmune diathesis and is frequently associated with diabetes mellitus: this triad of endocrinopathy was present in twenty-eight of the one hundred and thirteen patients with Addison's disease and diabetes reviewed by Solomon et al (1965), and others (Blizzard et al, 1967; Frey et al, 1973) have confirmed the preponderance of idiopathic as opposed to tuberculous Addison's disease in these patients. From all the above studies, it has become clear that autoimmune adrenal disease is associated with insulin-dependent (IDDM) rather than non-insulin-dependent diabetes (NIDDM), sometimes very strikingly so: the large series of Irvine et al (1980a) found IDDM in thirty-two (10 per cent) of three hundred and twenty-one patients with Addison's disease as opposed to only six (2 per cent) with NIDDM and observed that the age/

age at diagnosis of IDDM was over a very wide range. Only half the patients had developed diabetes before the age of thirty-five years, which is quite unlike the expected age-related incidence of IDDM, and suggests that cases of diabetes with idiopathic Addison's disease are much more likely to need insulin irrespective of the age at which diabetes appears, even if diabetic control is initially achieved by oral hypoglycaemic drugs (Irvine et al, 1980a). It has also become clear that adrenal failure precedes pancreatic failure and vice versa in approximately equal numbers and that both diseases make a simultaneous clinical appearance in the minority of cases.

The association between diabetes and pernicious anaemia was first described in 1910 (Parkinson, 1910) and since then has been described by several investigators (Arapkis et al, 1963; Munichoodappa and Kozak, 1970; Ungar et al, 1968; Irvine et al, 1970). The prevalence of diabetes in patients with pernicious anaemia is said to be 7 per cent (Ungar et al, 1967), again well in excess of the figure for the general population, and conversely the prevalence of pernicious anaemia in selected diabetic populations has been variously estimated as 0.39 per cent (Chanarin, 1964), 4.0 per cent (Ungar et al, 1968) and 5.0 per cent (Irvine et al, 1970). The lower figures probably underestimate the true position as pernicious anaemia is often of latent form in diabetics and associated with intrinsic-factor antibodies, frequently suggested as the 'marker' of latent or frank pernicious anaemia, in only 50 - 60 per cent of cases (Ardeman and Chanarin, 1963; Irvine, 1965). An accurate estimate of prevalence is therefore only achieved when the clinician is prepared to screen all diabetics at risk (mainly middle-aged to elderly females, irrespective/

irrespective of anti-diabetic treatment) not only for intrinsic-factor autoantibodies but also for an adequate serum vitamin B12 level (Ungar et al, 1968).

Multiple reports have indicated an association between diabetes and thyroid disorders. The diseases in question comprise thyrotoxicosis, usually in young IDDM (Hayles et al, 1959; Perlman, 1961; Nerup et al, 1977; Nerup et al, 1980; Cudworth and Wolf, 1982); chronic or Hashimoto thyroiditis, in both children and middle-aged patients (Landing et al, 1963; Crome et al, 1967; Masi et al, 1965); and primary hypothyroidism (Solomon et al, 1965; Hecht and Gerschberg, 1968; Andreani, 1974; Ganz and Kozak, 1974). In a prospective study of thyroid function in a diabetic population, excluding patients known to have thyroid disease, it was found that 9.8 per cent had impaired thyroid reserve (as indicated by a raised plasma TSH level) and 2.6 per cent had primary hypothyroidism (as indicated by low plasma T4 plus raised plasma TSH). The prevalence of this previously unrecognised thyroid dysfunction was greatest in IDDM over the age of fifty years, in whom impaired thyroid reserve was recorded in 22 per cent and hypothyroidism in 6.6 per cent of patients (Gray et al, 1979). Both pernicious anaemia and thyroid disorders occur with significant frequency (3 and 9 per cent respectively) in the first-degree relatives of diabetic patients (Irvine et al, 1970).

#### 1/4 Serum autoantibodies to non-pancreatic antigens in diabetes mellitus

The clinical associations of diabetes with overt autoimmunity are impressive but perhaps even more striking is the high prevalence of certain organ-specific autoantibodies in diabetic sera. Numerous serological studies have been performed in diabetics, directed in particular/

particular to the detection of thyroid or gastric autoantibodies, and the results of some of the more extensive investigations are presented in table 1/5. These studies have been performed in young and elderly as well as in unselected (random) populations, patients with overt thyroid, adrenal or gastric disease have been excluded, and in most instances the results have been compared with those from large nondiabetic populations of comparable age and sex distribution. The overall prevalence of various autoantibodies in diabetics can therefore be calculated with fair accuracy from the pooled data of the major series (table 1/6) and more detailed information is given in the following explanatory notes:

#### Thyroglobulin autoantibodies

Most investigators agree that the prevalence of antibodies to thyroglobulin in diabetes (measured by the tanned cell haemagglutination technique) is marginally but hardly significantly increased over that in the nondiabetic population. In both groups the overall prevalence seems to lie between 7 and 10 per cent (tables 1/5, 1/6), and major deviations from this relatively narrow range are found only in selected population strata: for example, the high figures recorded by Maret and Bethaud (1965) in diabetics and controls were obtained from groups whose members were almost exclusively female and all aged over seventy years. Only one study (Simkins, 1968) has described a major difference between diabetics and controls (prevalence 10 and 4 per cent respectively) and the results obtained by this investigator may be partly explicable by the ethnic differences between the groups; Simkins examined mixed Caucasian/Negro populations but half the diabetics and only 29 per cent of the control group were Negroes.

#### Autoantibodies/



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 AND IS NOT TO BE USED FOR ANY OTHER PURPOSE

NAME (LAST, FIRST, MIDDLE)	DATE OF BIRTH	PLACE OF BIRTH	CITY	STATE	COUNTRY	RACE	RELIGION	EDUCATION	OCCUPATION	MILITARY SERVICE	REMARKS
JAMES EARL RAY	5/5/24	TAMPA, FLORIDA	TAMPA	FLORIDA	UNITED STATES	WHITE	METHODIST	HIGH SCHOOL GRAD	BUSINESSMAN	NONE	FUGITIVE
ALVIN KARPIS	8/30/1929	MILWAUKEE, WISCONSIN	MILWAUKEE	WISCONSIN	UNITED STATES	WHITE	CATHOLIC	HIGH SCHOOL GRAD	BANK ROBBER	NONE	DECEASED
EDWARD GEORGE BREMER	12/12/1908	MILWAUKEE, WISCONSIN	MILWAUKEE	WISCONSIN	UNITED STATES	WHITE	CATHOLIC	HIGH SCHOOL GRAD	BANK ROBBER	NONE	DECEASED
JOHN EDGAR HOOVER	1/10/1895	WASH DC	WASH DC	DISTRICT OF COLUMBIA	UNITED STATES	WHITE	METHODIST	BACHELOR'S DEGREE	FEDERAL BUREAU OF INVESTIGATION	NONE	LIVING
JAMES EARL RAY	5/5/24	TAMPA, FLORIDA	TAMPA	FLORIDA	UNITED STATES	WHITE	METHODIST	HIGH SCHOOL GRAD	BUSINESSMAN	NONE	FUGITIVE
ALVIN KARPIS	8/30/1929	MILWAUKEE, WISCONSIN	MILWAUKEE	WISCONSIN	UNITED STATES	WHITE	CATHOLIC	HIGH SCHOOL GRAD	BANK ROBBER	NONE	DECEASED
EDWARD GEORGE BREMER	12/12/1908	MILWAUKEE, WISCONSIN	MILWAUKEE	WISCONSIN	UNITED STATES	WHITE	CATHOLIC	HIGH SCHOOL GRAD	BANK ROBBER	NONE	DECEASED
JOHN EDGAR HOOVER	1/10/1895	WASH DC	WASH DC	DISTRICT OF COLUMBIA	UNITED STATES	WHITE	METHODIST	BACHELOR'S DEGREE	FEDERAL BUREAU OF INVESTIGATION	NONE	LIVING

TABLE 1/5 (OPPOSITE):

Prevalence of autoantibodies in sera from diabetic and nondiabetic  
(control) populations, reported by various authors

T/G = antibody to thyroglobulin  
T/C = antibody to thyroid cytoplasm  
PCA = antibody to gastric parietal-cell cytoplasm  
IF = antibody to gastric intrinsic-factor  
ANF = antibody to cell nuclei  
ADR = antibody to adrenal cortex

TABLE 1/5

Prevalence of autoantibodies in sera from diabetic and nondiabetic  
(control) populations, reported by various authors

REFERENCE	SUBJECTS (NO. STUDIED)	NUMBER (PER CENT) POSITIVE FOR:					
		T/G	T/C	PCA	IF	ANF	ADR
Pettit, Landing and Guest (1961) <sup>a</sup>	Diabetics (58)	-	13(22)	-	-	-	-
	Controls (229)	-	2(1)	-	-	-	-
Landing et al (1963)	Diabetics (225)	-	40(18)	-	-	-	-
Moore and Neilson (1963)	Diabetics (65)	6(9)	-	-	-	-	-
	Controls (65)	5(8)	-	-	-	-	-
	Diabetics (83)	-	14(17)	18(22)	-	-	-
	Controls (166)	-	7(4)	13(8)	-	-	-
Maret and Berthaux (1965) <sup>b</sup>	Diabetics (82)	27(33)	-	-	-	-	-
	Controls (110)	28(26)	-	-	-	-	-
Ungar et al (1968)	Diabetics (400)	-	-	84(21)	8(2)	-	-
	Controls (600)	-	-	65(11)	-	-	-
	Controls (1600)	-	-	-	5(<1)	-	-
Simkins (1968)	Diabetics (317)	32(10)	-	-	-	-	-
	Controls (424)	17(4)	-	-	-	-	-
Irvine et al (1970)	Diabetics (1054)	90(9)	170(16)	176(17)	-	11(1)	-
	Controls (871)	56(6)	68(8)	53(6)	-	9(1)	-
	Diabetics (380)	-	-	-	13(3)	-	-
	Controls (296)	-	-	-	1(<1)	-	-
Goldstein et al (1970) <sup>c</sup>	Diabetics (252)	22(9)		15(6)	-	-	0(0)
	Controls (340)	20(6)		4(1)	-	-	0(0)
Whittingham et al (1971)	Diabetics (400)	-	56(14)	84(21)	8(2)	88(22)	-
	Controls (400)	-	34(9)	40(10)	0(0)	110(27)	-
Nerup and Binder (1973)	Diabetics (133)	13(10)	27(20)	21(16)	-	3(2)	3(2)
	Controls (128)	10(8)	4(3)	7(5)	-	0(0)	0(0)
MacCuish and Irvine (1975) <sup>d</sup>	Diabetics (250)	23(9)	43(17)	45(18)	-	3(1)	-
	Controls (250)	18(7)	21(8)	20(8)	-	2(1)	-

a All subjects aged under 16 years.

b All subjects aged over 70 years.

c No distinction between antibodies to thyroglobulin  
and thyroid cytoplasm.

d Part of study described in chapter six of this thesis.

TABLE 1/6

Overall prevalence of autoantibodies in sera from diabetic and nondiabetic populations, calculated from the pooled data in table 1/4

SUBJECTS (NUMBER STUDIED)	NUMBER (PER CENT) POSITIVE FOR:					
	T/G	T/C	PCA	IF	ANF	ADR
Diabetics (1901)	191(10)	-	-	-	-	-
Controls (1848)	134(7)	-	-	-	-	-
Diabetics (2203)	-	363(16)	-	-	-	-
Controls (2044)	-	136(7)	-	-	-	-
Diabetics (2572)	-	-	445(17)	-	-	-
Controls (2755)	-	-	202(7)	-	-	-
Diabetics (1180)	-	-	-	29(2)	-	-
Controls (2296)	-	-	-	6(<1)	-	-
Diabetics (1837)	-	-	-	-	106(6)	-
Controls (1649)	-	-	-	-	121(7)	-
Diabetics (385)	-	-	-	-	-	3(1)
Controls (468)	-	-	-	-	-	0(0)

Abbreviations as in table 1/5

### Autoantibodies to thyroid and gastric parietal cytoplasm

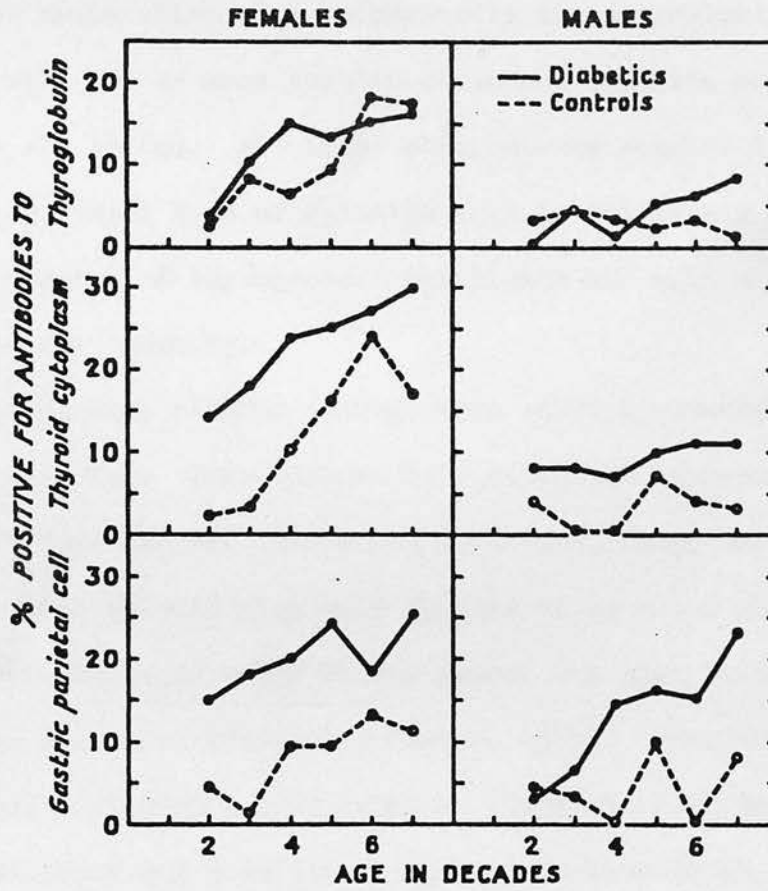
Antibodies to thyroid or gastric parietal cell (detected initially by complement fixation, latterly by the Coons indirect immunofluorescence technique) are conveniently considered jointly as their distribution is very similar in diabetic populations and indeed they may coexist in the same patient. Most investigators (table 1/5, figure 1/2) record a two- to four-fold increase in diabetics over the expected prevalence of either antibody in the general population but in selected groups, for example in juveniles, the difference may be even greater (Pettit et al, 1961). The combined effects of age, sex and anti-diabetic therapy on thyrogastic antibody prevalence have been carefully analysed in several studies and allow the following conclusions to be drawn:

- a) Thyrogastic antibodies are found most commonly in young (age forty or less), female, insulin-dependent diabetics.
- b) The prevalence of thyrogastic antibodies in older diabetics (i.e. above age forty) is much closer to that in control populations. Again the excess is mainly associated with insulin dependency and female sex.
- c) There is no significant difference in thyrogastic antibody prevalence between non-insulin-dependent diabetics and matched non-diabetic controls.
- d) The joint occurrence of thyroid and gastric antibodies is significantly more common in diabetics than nondiabetics.

Thus the marked difference in thyrogastic antibody prevalence between diabetics and (nondiabetic) controls is attributable to the insulin-dependent diabetic population. Moreover, similar studies have/



FIGURE 1/2



Percentage incidence of autoantibodies specific for thyroglobulin, thyroid cytoplasm and gastric parietal-cell cytoplasm in a series of 1032 diabetics and of 871 controls according to sex and age in decades.

From Irvine et al (1970)

have shown that thyroid and gastric antibodies, or overt autoimmune diseases, are significantly more frequent among first-degree relatives of diabetic children with thyrogastic antibodies than among relatives of antibody-negative children (Nissley et al, 1973; Bottazzo et al, 1978a). The association with autoimmunity is particularly striking in families with two or more insulin-dependent diabetic members (Bottazzo et al, 1978a). All these observations suggest that it is the insulin-dependent type of diabetes that is intimately associated with the possession of thyrogastic antibodies and with a familial background of autoimmunity.

Few studies have related thyrogastic antibody prevalence to duration of diabetes. Some workers have described increasing prevalence of thyrogastic antibodies with lengthening duration of disease, but this relationship only appears to be valid for insulin-dependent patients, aged under thirty years, who have been diabetic for more than ten years (Nerup and Binder, 1973). Conversely the most extensive published series found no clearcut relationship between antibody prevalence and duration of disease (Irvine et al, 1970). Whittingham et al (1971) applied a computer programme to carefully-matched populations and claimed to detect an actual decrease in thyrogastic antibody prevalence with increasing duration of insulin-dependent diabetes, the decrease being especially marked in diabetics of more than twenty years' standing. These authors offered the explanation that their findings reflected an unexpectedly high death rate among long-standing diabetics with thyrogastic antibodies, and further demonstrated that antibody prevalence in longterm insulin-dependent diabetics aged under forty is equal to that in the nondiabetic population/

population aged over sixty. Serologically, therefore, there is some suggestion that the longterm insulin-dependent diabetic population, especially female, has aged prematurely by at least twenty years, but how this phenomenon might contribute to an excess mortality amongst these patients is not clear. Apart from degenerative disease of the large blood vessels, there is no other convincing biological evidence of premature ageing in diabetes mellitus.

#### Autoantibodies to gastric intrinsic factor

Antibodies to intrinsic factor (IF), detected by a radio-immunoassay method using coated charcoal, are found much less commonly than parietal-cell antibodies in diabetes. Nonetheless their presence is of especial importance because of the strong association, mentioned earlier, with underlying pernicious anaemia; thus Ungar et al (1968) found IF antibody in eight of four hundred diabetics studied, three of whom had latent pernicious anaemia, while Irvine et al (1970) found IF antibody in thirteen of three hundred and eighty patients studied, latent pernicious anaemia being present in six of the nine patients who underwent additional investigations. The apparently low prevalence of IF antibody in the overall diabetic population (table 1/5) appears in correct perspective when it is appreciated that all investigators who have searched for this antibody are unanimous in indicating that IF antibodies are found only in middle-aged to elderly diabetics, almost exclusively female and predominantly insulin-dependent. In these selected population strata it has been estimated that the prevalence of IF antibody is between 4 and 5 per cent (Ungar et al, 1968; Irvine et al, 1970) and half the patients thus identified will have latent pernicious anaemia. These figures represent an increase of/

of at least four-fold over the expected prevalence of IF antibody in matched non-diabetic populations and are sufficiently impressive to reinforce the value of screening for IF antibody in the diabetic population at particular risk.

#### Autoantibodies to adrenal cortex

Few investigators have examined diabetic populations for adrenal antibodies (table 1/4). However it is worth noting that this antibody, which is detectable in 50 to 70 per cent of patients with idiopathic Addison's disease and is extremely rare outwith that context, was found by Nerup and Binder (1973) in three and by Gudworth et al (1980) in four insulin-dependent diabetics with no overt evidence of adrenal failure. The strong clinical associations of diabetes with Addison's disease have already been commented upon, and cell-mediated immunity against adrenal antigen has also been demonstrated in vitro in diabetics (Nerup and Bendixen, 1969).

#### Other autoantibodies in diabetes

The prevalence of antibodies which are tissue-specific rather than organ-specific, for example mitochondrial antibody and anti-nuclear antibody (ANF), does not appear to be increased in diabetic populations (table 1/5).

#### 1/5 Pathology of the endocrine pancreas in diabetes

A number of studies over the past two decades have described the pathological findings in the endocrine pancreas of both major types of diabetes mellitus. The major conclusions are summarised in table 1/7, which brings together the information from numerous histopathological studies using 'classical' staining techniques (e.g. the Gomori technique) and the more recent studies with immuno-cytochemical/



Pathology of the endocrine pancreas in insulin-dependent and  
non-insulin-dependent diabetics

AUTHORS	PATHOLOGICAL FEATURE	TYPE OF DIABETES MELLITUS STUDIED		
		Acute IDDM <sup>a</sup>	Chronic IDDM <sup>b</sup>	NIDDM <sup>c</sup>
Ogilvie (1964)	Weight of pancreas	Normal	Marked/moderate reduction	Normal
Gepts (1965)	Islet numbers	Reduced	Marked reduction	Normal/modest reduction
Warren et al (1966)	B cell numbers	Reduced by 90%	Reduced by 90%	Reduced by 10-20%
Doniach and Morgan (1974)	Normal islets	Absent	Absent	Predominant
Junker et al (1977)	Pseudoatrophic islets (A & D cells)	Predominant	Predominant	Absent
Orci et al (1976)	Hyperactive islets (mainly B cells)	Present	Absent	Absent
Gepts and DeMey (1978)	PP islet numbers	Increased	Increased	Normal
Gepts and LeCompte (1981)	'Insulinitis'	Present (50% cases)*	Absent	Absent

<sup>a</sup>Acute IDDM = Insulin-dependent, onset under age 22, death within 12 months of diagnosis. Most untreated at time of death.

<sup>b</sup>Chronic IDDM = Insulin-dependent, onset under age 30 (most under age 22). Insulin-treated for at least 2 years (average 18 years).

<sup>c</sup>NIDDM = Non-insulin-dependent. Onset over age 40 years.

\*21 of 42 cases reported by Gepts, Doniach and Morgan and Junker et al.



immunocytochemical techniques which allow reliable identification of the various hormone-producing cells within and outwith the islets of Langerhans. It is immediately apparent from table 1/7 that there are major differences between the changes observed in the pancreas in insulin-dependent and non-insulin-dependent diabetes: the histology of the gland in NIDDM is essentially normal, apart from a moderate reduction in total numbers of islets and B (beta) cell population. By contrast, IDDM is distinguished by a severe reduction in total islet numbers and the beta cell population is grossly reduced, in fact decimated. Many of the remaining islets are so small that they readily go undetected by light microscopy: formerly referred to as 'atrophic', these small islets are now known to be composed almost exclusively of A (alpha: glucagon-producing) and D (delta: somatostatin-producing) cells. They are termed 'pseudo-atrophic' islets and presumably reflect normal islets after loss of the beta cell population. Hyperactive islets are much rarer, found only in cases of short clinical duration and comprise mainly of hyperactive, degranulated beta cells of low insulin content; their significance is self-evident. The PP islets are entirely composed of irregular cords of columnar cells, sometimes called F cells, which secrete pancreatic polypeptide (PP) hormone; they seem to be more common in IDDM of long or short duration.

In the context of autoimmunity, the finding of insulinitis has excited particular speculation. The term 'insulinitis' was first coined by von Meyenburg (1940) to describe the inflammatory lesions in the islets of Langerhans of some diabetics, although such lesions had in fact been described by the early investigators of diabetic pathology/

pathology at the beginning of this century, well before the era of insulin treatment (Schmidt, 1902; Heiberg, 1911). The lesions of insulitis may be heavy, involving the majority of the islets, or light, involving only scattered islets. Affected islets are usually of the pseudo-atrophic type and infiltrated with lymphocytes, commonly round their periphery but sometimes throughout the islet. Large mononuclear cells, sometimes polymorphs and very rarely eosinophils may also be present in the infiltrate, but plasma cells have never been observed. The most common appearance seems to be of a 'halo' of lymphocytes just outside the capsule of the islet, extending a little way into the periphery of the endocrine tissue and probably cuffing capillaries in the islet stroma (Warren et al, 1966); more diffuse lymphocytic infiltration, over-running the whole islet, is less commonly encountered (Gepts, 1965). These lesions in human diabetes are quite distinct from the insulitis which occurs in some infants of diabetic mothers; the latter condition is characterised by an infiltrate which is predominantly of polymorphs, includes many eosinophils, and is without effect on pancreatic insulin production.

For many years insulitis was considered to be a very rare phenomenon, confined to a few cases of diabetes of recent onset in children (Warren and Root, 1925; LeCompte, 1958; Ogilvie, 1964). It was only briefly mentioned, or not at all, in many textbooks on pathology of diabetes and many pathologists have never seen a case (LeCompte and Legg, 1972). The finding by Gepts (1965) of insulitis in fifteen out of twenty-two young diabetics, i.e. two-thirds of the cases that he examined, was therefore in direct contrast to this classical opinion and since then there have been two further reported studies/

studies of insulinitis in diabetes. Doniach and Morgan (1973) were unable to find the lesion in any of nine young insulin-dependent diabetics who had died within a few weeks of diagnosis, or in four further cases who had been diabetic for longer periods but Junker et al (1977) found typical insulinitis in the pancreas of six of eleven young patients that they examined. In all, these three papers described the finding of insulinitis in twenty-one from a total of forty-two cases, i.e. a prevalence of 50 per cent, and this is probably as accurate a figure as can be arrived at in the modern clinical era of diabetic therapy.

Why was insulinitis considered to be so rare? There are probably several reasons. First, very few pathologists now have the opportunity to examine by modern techniques the pancreas in IDDM shortly after the onset of clinical disease, and of course this is a direct result of the advent of insulin therapy. Even the modern studies from which the prevalence of insulinitis has been estimated are comprised largely of material from pathological archives: all the specimens examined by Gepts, Doniach and Morgan were from persons who died between 1913 and 1930, i.e. before and shortly after insulin became available. Second, insulinitis is a transient lesion which has never been detected in IDDM who have died more than twelve months after the onset of disease. Its appearance is confined to IDDM of short duration, irrespective of the age of onset of the disease (LeCompte and Legg (1972) found it in two IDDM aged sixty-six and seventy years), and it has never been found in NIDDM. Third, insulinitis is a patchy lesion even when present; it may be absent from the bulk of the pancreas/

pancreas or may affect only scattered pseudo-atrophic islets. It has never been observed in the PP islets and is rarely seen in hyperactive islets. These correlations of islet morphology with insulitis are interesting insofar as they suggest that the mononuclear infiltrate is heaviest in the islets which have lost the highest proportion of beta cells and the inference might be drawn that the two phenomena are causally related.

Several theories have been advanced as to the cause(s) of insulitis, and by inference as to the cause of IDDM in cases where the lesion has been observed. Early workers considered that insulitis represented destruction of the islet cells by overstimulation or by some unidentified chemical cytotoxic agent (von Meyenburg, 1940). The former explanation seems improbable, and no similar lesions have ever been observed in other endocrine glands submitted to overstimulation but the latter suggestion is a possibility, to judge from the finding of insulitis and diabetes in mice treated by repeated injections of streptozotocin (Like et al, 1976, 1978). The observation that streptozotocin-induced insulitis can only be induced in certain strains of mice (Rossini et al, 1976) further suggests that specific genetically-determined immunological responses must be present before the lesion can develop and this may have important implications for human diabetes. The various animal models in which insulitis has been induced by immune mechanisms, or observed to occur spontaneously, are considered below while direct and indirect evidence for viral infection as a cause of insulitis is discussed in chapter seven. In passing, it can be said that there has been a very natural tendency to compare insulitis with the organ-specific/



specific chronic inflammatory lesions found in classical thyrogastric and adrenal autoimmune diseases but the analogy does not extend to the presence of plasma cells, which have never been described in the insulinitis lesion in man.

#### 1/6 Animal models of experimental immune diabetes

The immunological methods which have been used for the experimental induction of insulinitis (and diabetes mellitus) are detailed in table 1/8 while the various animal models in which these methods have been applied are summarised in table 1/9. It will be seen that both passive (e.g. injection of heterologous anti-insulin serum) and active immunisation procedures (e.g. injection of heterologous or homologous insulin and endocrine pancreas) have been successful in producing insulinitis in a variety of species. Experimentally induced lesions of the islets of Langerhans, which to some extent deserve the term 'insulinitis', were observed first by Lacy and Wright (1965). These authors injected guinea-pig antiserum against bovine insulin into rats, producing a diabetic state by neutralisation of endogenous insulin. Morphological studies in a few animals revealed mononuclear and eosinophilic cells with some islets but the main histological feature was a diffuse exocrine pancreatitis. Shortly afterwards Logothetopoulos and Bell (1968) used the same technique to induce very similar lesions of the islets in mice. These experiments led to an acute form of insulinitis but Freytag (1972) later demonstrated that heterologous antiserum in mice could also induce a chronic form of insulinitis, typified by the infiltration of mononuclear cells around and within the islets and by the virtual absence of eosinophils.

In/



TABLE 1/8

Immunological methods used for the induction of insulinitis  
in experimental animals

Principle	Method	Species
Passive Immunisation	Intravenous injection of guinea-pig anti-bovine-insulin serum	( Rats Mice
	Transfer of lymphoid cells from insulin-immunised animals to immunosuppressed young animals	Guinea-pigs
Active Immunisation	Subcutaneous injection of homologous or heterologous insulin + Freund's adjuvant	Cattle Sheep Rabbits Guinea-pigs
	Subcutaneous injection of homologous or heterologous endocrine pancreas + CFA*	( Rats Mice

\*CFA = complete Freund's Adjuvant

TABLE 1/9

The induction of insulinitis in experimental animals  
by passive and active immunisation procedures

Method	Species	Authors	Findings
Injection of heterologous anti-insulin serum	Rat	Lacy and Wright (1965)	Diffuse exocrine pancreatitis
	Mouse	Logothetopoulos and Bell (1968)	Acute transient insulinitis (mainly eosinophils). Carbohydrate intolerance.
	Mouse	Freytag (1972)	Chronic insulinitis, mainly monocytes.
Lymphoid-cell transfer from insulin-immunised to immunosuppressed young animals	Guinea-pig	Titlbach et al (1975)	Insulinitis (mononuclear) Beta cell degranulation. Carbohydrate intolerance.
Injection of heterologous insulin in FA	Cattle	Renold et al (1964) LeCompte et al (1966)	Chronic insulinitis (mononuclear).
	Sheep	Federlin et al (1968)	Fibrosis of some islets.
	Rabbit	Toreson et al (1964) Grodsky et al (1966) Lee et al (1969) Kloppel et al (1972, 1976).	Exocrine pancreas normal. Humoral anti-insulin antibody production Diabetes in a few animals
Injection of homologous insulin in FA	Cattle	Renold et al (1964) LeCompte et al (1966)	Identical to experiments with heterologous insulin.
	Sheep	Federlin et al (1968)	
Injection of homologous and heterologous endocrine pancreas in CFA	Rat	Nerup et al (1973a)	Insulinitis (mononuclear). B cell degeneration. Exocrine pancreas normal.
		Andersen et al (1974)	Antipancreatic cellular hypersensitivity (by LMT) Transient diabetic state.

In the context of autoimmunity against the endocrine pancreas, the experiments by the group of Renold (Renold et al, 1964; LeCompte et al, 1966; Federlin et al, 1968) were of particular interest and importance. These workers showed that the injection of homologous insulin in cattle or sheep, over periods ranging from four months to two years, frequently results in pathological changes in the pancreatic islets which strongly resembled the lesions of insulitis found by Gepts (1965) in human diabetes. In affected animals the lesions were confined to islet tissue, the exocrine pancreas being uninvolved. The infiltration was typically mononuclear, mainly mature lymphocytes, and in some islets there was extensive fibrosis (LeCompte et al, 1966). The appearance of insulitis was consistently accompanied by the development of humoral anti-insulin antibodies, while the presence of coexisting cell-mediated immunity to insulin was shown by the appearance of strongly positive delayed-type skin hypersensitivity to injected insulin.

A difficulty in accepting the results of Renold et al as applicable to the development of insulitis in man was the relative rarity with which insulitis in animals was accompanied by carbohydrate intolerance. This objection was overcome in the rodent model of experimental immune insulitis described by Nerup and his colleagues (Nerup et al, 1973a; Andersen et al, 1974). These workers prepared suspensions of homologous endocrine pancreas by using the collagenase dissection technique to isolate the islets of murine pancreas and admixing the tissue in complete Freund's adjuvant: they found that the injection of this preparation into inbred mice was followed by the appearance of classical insulitis, glucose intolerance and cell-mediated antipancreatic hypersensitivity (by the LMT) within fourteen days of injection. The carbohydrate/

carbohydrate intolerance and morphological changes were transient in nature, disappearing within a month of the initial injection; nonetheless the findings have relevance to the hypothesis of autoimmune diabetes in man.

The most obvious objection to extrapolating the results of all the above studies to man has been the artificial nature of the procedures used for immunisation of experimental animals. Perhaps more relevant for the aetiology of human IDDM are the recent experiments in which diabetes has been induced in rodents by common viruses or by exposure to noxious chemicals, or the fascinating animal model of spontaneous autoimmune diabetes which has been lately described. These models are summarised in table 1/10. It will be seen that diabetes mellitus with selective beta cell loss has now been induced in mice by three different viruses (EMC virus, reovirus and Coxsackie virus). The relevance of these observations to the aetiology of IDDM in man is considered more fully in chapter seven (section 7/2) but for the present, it should be noted that susceptibility to the effects of viral infection is largely dependent upon the strain of the mice under test (Boucher et al, 1975), thus indicating immediately that there is a genetic variation in immunologically-mediated response to a viral infection. The animal model (table 1/10) in which insulinitis and severe diabetes was induced by repeated small doses of streptozotocin might be thought to have little or no relevance to the aetiology of human diabetes with insulinitis: however streptozotocin is a nitrosamine compound with molecular structure similar to a widely-used pesticide which was introduced into agricultural use about ten years ago/



TABLE 1/10

Insulinitis and diabetes mellitus induced by viral infection, toxic chemicals or heredity (spontaneous diabetes) in mice and rats

Agent	Authors	Findings
EMC-virus Reovirus type 3 Coxsackie virus type B4	Craighead et al (1975, 1981) Onodera et al (1978) Yoon et al (1978)	Insulitis (mononuclear). Beta cell degeneration. Exocrine pancreas normal. Prolonged diabetic state.
Streptozotocin (40 mg/kg) in repeated dosage	Rossine and Like (1976) Like et al (1977, 1978) Rossini et al (1980)	Insulitis (mononuclear). Selective beta cell degeneration. Proliferation of type-C virus in beta cells. Severe insulin- dependent diabetes mellitus.
Outbred strain of Wistar rate (BB/W rat) - approx. 30% of colony	Nakhooda et al (1976, 1978) Dyrberg et al (1982) Like et al (1982)	Intense insulitis (mononuclear). Gross beta cell loss. Severe insulin-dependent diabetes. Humoral and cell-mediated autoimmunity. Extrapancreatic autoimmune phenomena.



ago and has since been implicated as a causative factor in at least two hundred cases of human IDDM (Prosser et al, 1978; Karam et al, 1979). Thus it is not impossible that toxic chemicals might cause a proportion of cases of immunologically-mediated beta cell destruction in man. As with virus infections, there are marked intra-strain variations in murine host susceptibility to streptozotocin, correlated with the haplotype of the animals, which again indicates a genetically-determined immune response to an environmental factor.

The last and perhaps the most interesting animal model of insulinitis and diabetes is the syndrome of severe, insulin-dependent diabetes which appeared spontaneously in a commercial outbred colony of Wistar-derived albino rats (now termed the Bio Breeding/Worcester or BB/W rat; table 1/10). The syndrome has been extensively studied (Nakhooda et al, 1977, 1978) and its major characteristics are as follows: about 30 per cent of genetically susceptible animals develop the syndrome at an early age (two to four months), both sexes are equally affected and the animals are lean. There is an abrupt onset of diabetes and 99 per cent of affected animals need daily insulin injections to sustain life. The histology of the pancreas shows a profound insulinitis, with rapid beta cell destruction and the appearance of pseudo-atrophic islets (as in human IDDM) composed of A and D cells. The evidence for cell-mediated autoimmunity in the BB/W rat comprises the presence of insulinitis; prevention or modification of the diabetes by treatment with antilymphocytic serum (ALS); prevention of diabetes by neonatal thymectomy or by bone marrow transplant from normal rats (Like et al, 1979, 1981, 1982; Naji et al, 1981). The evidence for humoral autoimmunity comprises the demonstration/

demonstration of immunoglobulin bound to the surface of pancreatic islet cells (islet cell surface antibody: see chapter two) by a protein-A radioligand assay (Dyrberg et al, 1982). Finally, affected animals show evidence of extra-pancreatic autoimmunity in the form of lymphocytic thyroiditis, closely resembling Hashimoto thyroiditis in man, and the presence of circulating autoantibodies to thyroglobulin, thyroid follicular cell, gastric parietal cell, smooth and skeletal muscle, and cell nuclei (Like et al, 1982). This fascinating animal model represents the closest approach at the present time to a model of polyendocrine autoimmune disease in which IDDM is the leading component and has developed through cell-mediated autoimmune mechanism. Studies of the BB/W rat have obvious implications for the study and manipulation of immune mechanisms in human insulin-dependent diabetes, although it appears that the diabetic syndrome in these animals is not dependent upon any recognised infectious agents (Rossini et al, 1979).

#### 1/7 Summary and conclusions

The studies reviewed in this chapter may be summarised as follows:

- a) Insulin-dependent diabetes is intimately related to the classical organ-specific autoimmune diseases, both on an individual and a familial basis.
- b) Thyrogastic autoantibodies are significantly more common in insulin-dependent diabetics than age- and sex-matched nondiabetics.
- c) There is no obvious connection between non-insulin-dependent diabetes and overt or serological evidence of endocrine autoimmune disease.

d/

d) Insulinitis (mononuclear infiltration of the pancreatic islets) is not found in non-insulin-dependent diabetics or in any patient with disease duration of longer than (about) one year. Its presence is confined to recently diagnosed insulin-dependent patients and it may be found in about half of such cases.

e) In animal models it has proved possible to induce insulinitis and diabetes by various immunisation procedures, viral infection, exposure to toxic chemicals and selective breeding. Genetic factors are of demonstrable importance in determining the response of individual strains of animals to environmental factors and hence of determining susceptibility to the insulinitis/diabetes syndrome.

The above studies have therefore provided good indirect evidence to associate insulin-dependent diabetes mellitus with autoimmunity. Studies which have searched for more direct evidence of humoral and cell-mediated autoimmunity in diabetes are presented in succeeding chapters.

## CHAPTER TWO

## STUDIES OF HUMORAL AUTOIMMUNITY IN DIABETES MELLITUS

2/1 Introduction and purpose of studies

At the time when this work commenced, one major difficulty in applying the autoimmune hypothesis to diabetes mellitus had been the failure to find humoral autoantibodies, directed against the endocrine pancreas, in sera from diabetics. Early investigators, using haemagglutination, gel-diffusion and precipitin techniques for antibody detection, claimed to find antibodies against the pancreas in the sera of a significant proportion of the diabetics that they tested, as well as in other forms of pancreatic disease (Murray and Thal, 1960; Fonkalsrud and Longmire, 1961). For example, Murray and Thal reported the presence of such antibodies in the sera of seven (12%) of sixty-one diabetics and forty-five (90%) of fifty patients with chronic pancreatitis or pancreatic cancer. These results were not confirmed subsequently and were probably due to methodological artefacts. Villavicencio et al (1965) used the same techniques as Murray and Thal and failed to find any anti-pancreatic antibodies in the sera of one hundred and forty-two diabetics and sixteen patients with pancreatitis. These authors noted that Murray and Thal's antigen was prepared at 10000 rpm and that Fonkalsrud spun his pancreatic extract at 2000 rpm; at these low speeds there exists the possibility of spurious positive gel-diffusion tests because of incomplete removal of haemoglobin, and the haemagglutination test cannot be performed because of lysis of the red blood cells. Later attempts to detect antipancreatic antibody in diabetes by the indirect immunofluorescence technique were equally disappointing (Irvine/



(Irvine et al, 1970; Nerup and Binder, 1973; Doniach, 1974a).

Since the detection of a humoral autoantibody was central to the whole concept of autoimmunopathology in diabetes, it was decided to make a further search for antipancreatic antibodies in this disease. The time was propitious, since quantities of fresh human pancreas to use as tissue substrate were becoming available through the increasing volume of cadaveric renal transplantation surgery, and the quality of technical equipment for immunofluorescence microscopy was steadily improving. From the earlier literature it was apparent that previous investigators had examined very few diabetics with coexistent autoimmune endocrinopathies, i.e. those patients who might be expected to show the strongest expression of an autoimmune diathesis, and accordingly it seemed logical to commence the investigation in this selected group of patients.

## 2/2 Selection of diabetics for study

For the initial study, sera from a total of one hundred and five diabetics and controls were examined, the subjects being subdivided into the following groups:

### Group A: Diabetics with coexistent overt autoimmune disease

This group comprised twenty insulin-dependent diabetics with coexistent idiopathic Addison's disease. All had circulating adrenal antibodies and all but two were women. Apart from adrenal failure, nine had one or more additional autoimmune disease (thyrotoxicosis, Hashimoto thyroiditis, primary hypothyroidism, pernicious anaemia, premature ovarian failure), accompanied by the appropriate circulating antibodies.

### Group B: Diabetics with thyrogastic autoantibodies

This/



This group comprised twenty insulin-dependent diabetics with circulating thyroid and/or gastric parietal-cytoplasmic antibodies, but no evidence of overt autoimmune disease. These patients were matched as closely as possible (but not exactly) with the patients in group A for age, sex and duration of diabetes.

#### Group C: Diabetics with negative autoantibodies

This group comprised twenty-five insulin-dependent diabetics, with no overt autoimmune disease and no organ-specific antibodies in the serum. Patients in this group were closely comparable to those in groups A and B in respect of age, sex and duration of diabetes.

#### Group D: Nondiabetic controls with negative autoantibodies

This group comprised forty healthy nondiabetic controls with no overt autoimmune disease and no organ-specific antibodies in the serum. They were obtained from the same sources as the normal subjects used in the study of nonpancreatic antibodies in diabetes (chapter six) and were broadly comparable, in age and sex, to the subjects in the three diabetic groups above.

### 2/3 Methods used for detection of autoantibodies

#### Detection of islet-cell antibodies

Fresh human pancreas was used as the tissue substrate for these experiments, and was obtained immediately after death from cadaveric renal transplant donors of blood group O. Group O pancreas was found to be essential since the use of pancreas from group A or B donors frequently gave immunofluorescence of acinar cells, thus making the detection of any possible islet-cell fluorescence extremely difficult; the phenomenon occurs because the cells of the exocrine pancreas are analogous/

analogous to those of the gastric mucosa in possessing membrane antigens of the blood group system and in synthesising blood group substances in intracellular microsomes. The specimens were kindly supplied by Mr. A.C.B. Dean, Consultant Surgeon, Royal Infirmary, and Mr. A. McL. Jenkins, Lecturer in Surgery, University of Edinburgh. The tissue was snap-frozen immediately after removal, stored in small blocks at  $-40^{\circ}\text{C}$ , and cut as required into thin (5-7 micron) sections on a cryostat. All tests were performed on air-dried, unfixed sections. Islet-cell antibodies were detected by the Coons indirect immunofluorescence technique (Appendix 1/2), using antihuman IgG fluorescein-isothiocyanate (FTC) conjugate (Wellcome Reagents) at a 1:14 dilution. All sera were initially tested undiluted, but those giving positive results were subsequently retested in serial dilutions to establish the antibody titres. Positive sera were further tested with anti-IgA and anti-IgM conjugates to ascertain the immunoglobulin class of the antibodies, and complement-fixing ability was studied with an anti-B1C conjugate (Boehringer Corp). Experiments were also undertaken to confirm that the fluorescence observed with anti-IgG conjugate could be abolished by pretreatment of the pancreatic sections with unlabelled anti-human IgG.

#### Other autoantibody studies

Using the techniques described in chapter six and Appendix I, all sera were tested for antibodies to thyroglobulin, thyroid cytoplasm, gastric parietal-cell cytoplasm, cell nuclei and mitochondria. In addition sera from patients in group A with premature ovarian failure were examined for antibodies reactive to extra-adrenal steroid-producing cells (ovary, testis and placenta); this investigation was kindly undertaken/



undertaken by Dr. E.W. Barnes using the method of Irvine et al (1969). Serum from the single patient with pernicious anaemia was examined for intrinsic-factor-antibody 1 by radioimmunoassay using albumin-coated charcoal as described by Irvine et al (1968).

#### 2/4 Detection and general properties of pancreatic islet-cell antibody

##### Islet-cell antibodies

Cytoplasmic immunofluorescence of the pancreatic islets was detected in five of the hundred and five sera tested, all from patients in group A (table 2/1). In each case the fluorescence seemed to involve the islet cells diffusely, being present in A (alpha) and D (delta) cells in addition to B (beta) cells (figure 2/1). Immunofluorescence was detected using undiluted serum only in one case, the antibody titre ranging from 1:4 to 1:128 in the other four sera (table 2/2). Some variation in the intensity of fluorescence was observed with the two strongest sera, but in all cases the islets stood out brightly from surrounding exocrine tissue. Islet-cell antibodies were complement-fixing as shown by staining with addition of fresh human serum followed by anti-B1C conjugate and in all cases were of IgG class, immunofluorescence being negative with anti-IgA and anti-IgM conjugates. It was also possible to abolish the fluorescence by pretreatment of the pancreatic sections with unlabelled anti-IgG. With negative sera the pattern of fluorescence was entirely different (figure 2/2): the islets either merged with surrounding exocrine tissue or were only distinguished with difficulty, and any fluorescence observed was either an orange-pink autofluorescence or occasional faint cytoplasmic fluorescence, sporadically distributed in both endocrine and exocrine tissue.

##### Other/

TABLE 2/1

Antibodies to pancreatic islet-cell detected by immunofluorescence  
in 65 diabetic patients and 40 normal controls

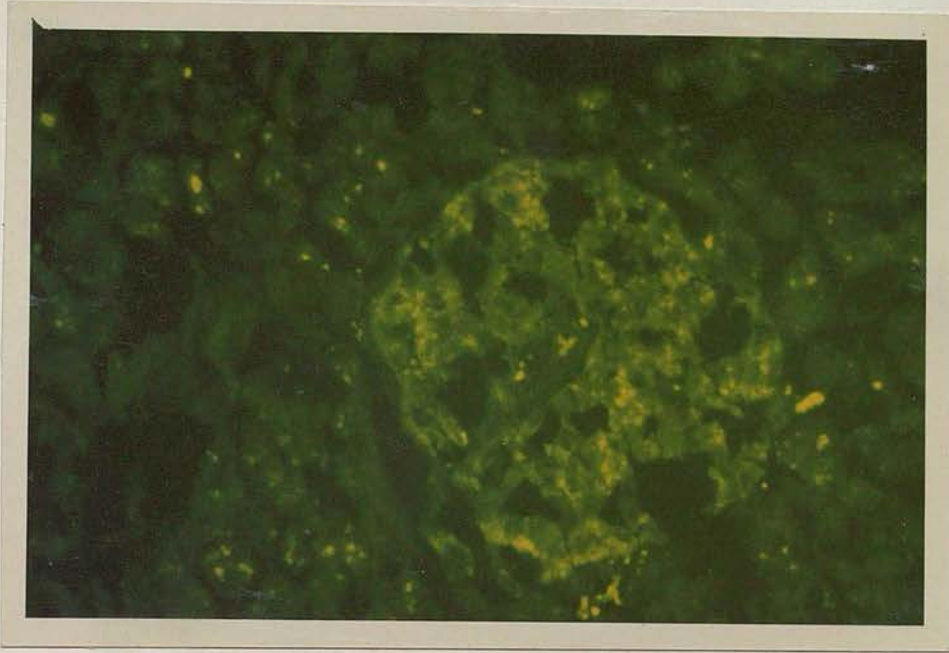
Group	Patients tested	Islet-cell antibody	
		Positive	Negative
A	Diabetics with Addison's disease	5	15
B	Diabetics with thyrogastric antibodies	0	20
C	Diabetics with negative autoantibodies	0	25
D	Controls with negative autoantibodies	0	40
	Total	5	100

See text for explanation of groups

From MacCuish et al (1974a).



FIGURE 2/1



Pancreatic islet-cell antibody in diabetes mellitus: cryostat section of human pancreas treated with serum of a diabetic patient, followed by antihuman IgG-FITC conjugate, showing cytoplasmic fluorescence over the islet of Langerhans. Fluorescence is apparent in all cell types in the islet; the exocrine pancreas is unaffected.

(Photographed with Leitz Orthomat equipment: Ektachrome EH135B Film)



TABLE 2/2 (OPPOSITE):

Clinical and serological findings in five diabetics with  
autoantibodies to pancreatic islets

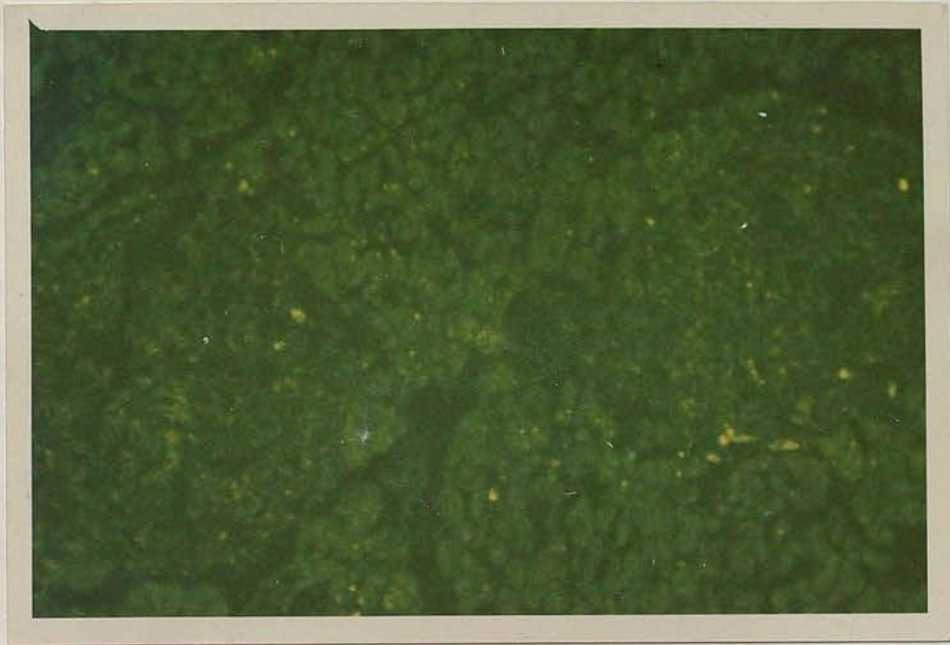
Thg = antibody to thyroglobulin  
TC = antibody to thyroid cytoplasm  
GPC = antibody to gastric parietal-cell cytoplasm  
IF = antibody to intrinsic factor  
ANA = antibody to cell nuclei  
Mito = antibody to mitochondria  
Plac = antibody to placenta

+ = antibody present, titre not determined  
- = antibody not present  
NT = serum not tested for antibody

Clinical and serological findings in five diabetics with  
autoantibodies to pancreatic islets

Age	Sex	Clinical Conditions	Age of Onset (yrs)	Autoantibodies										
				Islets (titre)	Adrenal (titre)	Thg (titre)	TC	GPC	IF (titre)	ANA	Mito	Ovary	Plao.	Testis
44	F	Thyrototoxicosis Diabetes mellitus Addison's disease Amenorrhoea	22 30 36 36	128	4	-	-	+	-	-	-	+	+	+
43	F	Amenorrhoea Pernicious anaemia Primary hypothyroidism Addison's disease Diabetes mellitus	28 33 40 40 43	64	8	250	+	+	94 u/ml	-	-	+	+	+
33	F	Hashimoto thyroiditis Addison's disease Diabetes mellitus	29 29 31	8	16	25000	+	+	-	-	-	NT	NT	NT
32	F	Diabetes mellitus Addison's disease	26 31	4	8	-	-	-	-	-	-	NT	NT	NT
45	F	Oligomenorrhoea Primary hypothyroidism Diabetes mellitus Addison's disease	30 31 44 45	Undilute serum only	+	-	+	-	-	-	-	+	+	-

FIGURE 2/2



Antibody-negative serum in diabetes mellitus. The pancreatic section has been prepared and treated exactly as for figure 2/1 but the test serum does not contain islet-cell antibody. The islets of Langerhans and the exocrine pancreas are both clearly visible in the photograph: neither type of tissue shows specific fluorescence.

(Photographed as for figure 5/1)



### Other autoimmune lesions in patients with islet-cell antibodies

The clinical and serological findings in the five cases with islet-cell antibodies are presented in table 2/2. As stated, all were diabetics with idiopathic Addison's disease and four had additional overt autoimmune disease or diseases. The three patients with premature ovarian failure had a multiplicity of steroid-cell antibodies reacting with different antigens in ovary, placenta and testis, typical of the serological findings in this polyendocrine syndrome (Irvine et al, 1969).

### 2/5 Properties of the pancreatic islet-cell antigen-antibody system

Using the above five positive sera as reference standards, some initial investigations into the nature of the islet-cell antigen-antibody system were undertaken. Air-dried pancreatic sections were exposed to a variety of fixatives, reagents, proteolytic enzymes, etc. before and after treatment with antibody-containing sera. The results (table 2/3) indicate that the activity of pancreatic islet-cell antigen is destroyed by reagents that separate lipids as much as by proteolytic enzymes; the general properties seem strikingly similar to those of the thyroid-cell or gastric parietal-cell autoimmune systems, as summarised by Doniach (1974b). These preliminary findings do not obviate the need for formal characterisation of pancreatic islet-cell antigen(s) by absorption and subcellular fractionation studies, but at least suggested by analogy that this antigen too may be microsomal in nature.

### 2/6 The prevalence of islet-cell antibody in diabetes, autoimmune disease and the normal population

Having established the existence of an islet-cell antibody in diabetes/

TABLE 2/3

Behaviour of the pancreatic islet-cell antigen-antibody system when exposed to various fixatives, etc.

<u>The Pancreatic Islet-Cell Antigen-Antibody System</u>	
<u>Destroyed by:</u>	<u>Unaffected by:</u>
Trypsin	Lipase
Papain	Phospholipase C
Alcohols	Alkali
Heat (100°C)	Acetone
Gluteraldehyde	EDTA
Detergent:	Detergent:
Lubrol-W	Tween-20
Formaldehyde	



diabetes, and having determined some of its characteristics, an investigation was undertaken to estimate the prevalence of this antibody in a larger diabetic population, as well as in nondiabetics with known autoimmune disease. For this purpose the initial series of one hundred and five sera described in section 2/2 of this chapter was extended to include the examination of serum samples from a total of nine hundred and three subjects, subdivided into the following groups:

#### Diabetic with coexistent autoimmune disease

This group comprised forty-six diabetics (thirty-five women, eleven men), all of whom had one or more coexistent autoimmune disease. There were twenty-one patients with idiopathic Addison's disease, which included the initial twenty patients described in section 2/2. Pernicious anaemia coexisted with diabetes in six patients, all of whom were on maintenance treatment with vitamin B12 injections. Thyroid autoimmune disease was present in the remaining nineteen patients: ten had primary hypothyroidism and were receiving L-thyroxine replacement therapy; nine were thyrotoxic, being treated with carbimazole, a therapeutic dose of  $^{131}\text{I}$ odine or (in one case) by partial thyroidectomy.

Thirty-eight of the patients in this group were insulin-dependent diabetics, the remainder being controlled by dietary carbohydrate restriction or oral hypoglycaemics (sulphonylurea or biguanide).

#### Diabetic with thyrogastric autoantibodies

This group comprised one hundred and seven diabetics (seventy women, thirty-seven men), all with thyroid cytoplasmic and/or gastric parietal-cell cytoplasmic antibodies in the sera but none with overt autoimmune disease. Forty-five of these patients had been identified by/

by the study described in chapter six and sixty-two had been identified during the extensive survey of Irvine et al (1970). Seventy-eight were insulin-dependent diabetics, the remainder being controlled by dietary carbohydrate restriction or oral hypoglycaemics.

#### Diabetic with negative autoantibodies

This group comprised two hundred diabetics (one hundred and fifty women, fifty men) with no overt autoimmune disease, no personal history of autoimmunity and no organ-specific antibodies in the serum. The majority of these patients had been identified by the study described in chapter six; one hundred and fifty were insulin-dependent diabetics, the remainder being controlled by dietary carbohydrate restriction or oral hypoglycaemics.

#### Nondiabetic with pernicious anaemia/atrophic gastritis

There were fifty patients (forty-five women, five men) in this group. In forty-six the diagnosis of pernicious anaemia had been made by the usual criteria (anaemia, low serum vitamin B<sub>12</sub>, megaloblastic bone marrow, achlorhydria, low Schilling test corrected with intrinsic factor); all these patients were being treated with regular injections of vitamin B<sub>12</sub> and all had circulating gastric parietal-cell and/or intrinsic factor antibodies. The remaining four patients in this group had the autoimmune type of atrophic gastritis as defined by Goldstone et al (1973).

#### Nondiabetic with Hashimoto thyroiditis

The fifty patients in this group were all women and all were attending the Endocrine Clinic at Edinburgh Royal Infirmary. Histological confirmation of the diagnosis was available in eighteen patients; in a further thirty the diagnosis was made on the basis of

a goitre and characteristic serological findings (high titres of circulating thyroid cytoplasmic and/or thyroglobulin antibodies). The remaining two patients had presented with a goitre and hypothyroidism in middle age and on clinical grounds were considered to have Hashimoto thyroiditis, although the antibody titres were low. All but four of the patients in this group were on long-term treatment with L-thyroxine.

#### Nondiabetic with idiopathic Addison's disease

The hundred patients (seventy-two women, twenty-eight men) in this group with idiopathic (autoimmune) Addison's disease had all been identified in the studies of Irvine and Barnes (1974, 1975), and all had circulating adrenocortical antibodies. They were receiving conventional steroid replacement therapy and thirty had polyendocrine autoimmune disease (premature gonadal failure, thyrogastric autoimmunity, idiopathic hypoparathyroidism, or a combination of these syndromes) in addition to adrenal failure.

#### Control subjects

This group comprised three hundred and fifty healthy nondiabetic subjects (two hundred women, one hundred and fifty men) with no overt autoimmune disease, no personal history of autoimmunity and no organ-specific antibodies in the serum. The majority of these controls had previously been used for the studies described in chapter six and were obtained from the sources mentioned in that chapter.

#### Detection of islet-cell antibodies

Islet-cell antibodies were detected as described in section 2/3 of this chapter. All sera giving positive results were tested using at least two different blocks of human group O pancreas as tissue substrate/

substrate.

## Results

The prevalence of islet-cell antibody in the various diabetic and nondiabetic subgroups is summarised in table 2/4. The antibody was found in a total of fifty-one diabetics. Fourteen (eleven women, three men, all insulin-dependent) had one or more major overt autoimmune disease in addition to diabetes: seven had idiopathic Addison's disease (including the five detailed in table 2/2), two had pernicious anaemia, four had primary hypothyroidism and one was thyrotoxic. The overall prevalence of islet-cell antibody in this selected group of forty-six patients was 30% and nine of the antibody-positive subjects were aged forty years or younger.

Nineteen diabetics with islet-cell antibody (twelve women, seven men, all but one insulin-dependent) had circulating thyrogastric antibodies but no clinical autoimmune disease. The overall prevalence of the antibody in this group of one hundred and seven patients was 18% and thirteen of the antibody-positive subjects were aged forty years or younger.

Islet-cell antibody was found in the serum of eighteen of two hundred diabetics who had no other clinical or serological evidence of autoimmune disease. The overall prevalence of antibody in this group was 9% and twelve of antibody-positive subjects were aged forty years or younger. All but two had insulin-dependent diabetes, the exceptions being one woman and one man who were being treated with the oral hypoglycaemic drug chlorpropamide.

With regard to nondiabetics with overt autoimmune disease, the antibody was found in only two such patients - i.e., an overall prevalence/



TABLE 2/4

The prevalence of pancreatic islet-cell antibody in diabetics with and without autoimmunity, in nondiabetics with autoimmune disease and in normal subjects

Group of subjects tested	Islet-cell antibody	
	Negative	Positive
Diabetic with coexistent autoimmune disease	32	14(30%)
Diabetic with thyrogastric autoantibodies	88	19(18%)
Diabetic with negative autoantibodies	182	18(9%)
Nondiabetic with PA/atrophic gastritis	49	1(2%)
Nondiabetic with Hashimoto thyroiditis	50	0
Nondiabetic with Addison's disease	99	1(1%)*
Normal controls	350	0

(see text for detailed description of the groups)

PA = pernicious anaemia

\*Subsequently developed diabetes (see text)



prevalence of 1%. One was a woman, aged thirty-four years with pernicious anaemia; no information on her response to glucose tolerance testing was available. The other was a forty-six year old woman with idiopathic Addison's disease and thyrotoxicosis. This patient presented with typical symptoms of thirst and polyuria six months after her serum was tested and subsequently developed insulin-dependent diabetes mellitus.

Islet-cell antibody was not found in any of three hundred and fifty normal subjects.

## 2/7 Discussion of results

The five examples of pancreatic islet-cell antibody (ICA) detected in the initial study described in this chapter (section 2/4; table 2/2) were published in detail at the end of 1974 (MacGuish et al, 1974a). Four weeks previously the presence of an islet-cell antibody in sera from thirteen patients had been reported by Professor Deborah Doniach's group at the Middlesex Hospital (Bottazzo et al, 1974): from a comparison of the two reports it was clear that the findings were identical, and that the existence of a new antibody directed against the endocrine pancreas had thus been confirmed by two independent centres. The first question to ask, therefore, was why this particular antibody had remained undetected for so long. Several hundred diabetic sera had previously been tested by workers interested in this problem, using the same immunofluorescence technique but without success; however these studies either specifically excluded patients with clinically coexistent diabetes and autoimmune disease (Irvine et al, 1970; Nerup and Binder, 1973) or tested unselected patients, many of whom were insulin-independent (Doniach, 1974a). In retrospect it is now clear that these populations/

populations were unlikely to have contained antibody- positive patients and were markedly different to the populations investigated by Bottazzo et al (1974) and by the author. Moreover, earlier workers did not fully appreciate that blood group substances are normally secreted by the pancreatic acini, so that group A or B organs give substantial fluorescence with many sera (Lendrum and Walker, 1975), while fresh human pancreas from group O donors was not readily available until cadaveric renal transplantation became established in the early 1970's. Finally, pancreatic islet-cell antibody is present in only low titres in most subjects (the highest titre recorded in the present series being 1:256), and produces a comparatively weak immunofluorescence by comparison with the strikingly vivid cytoplasmic staining exhibited by thyroid or gastric microsomal antibodies. Early UV microscopes, with dark-field condensers and light reflected from below by a mirror, had a much lower visibility than present-day instruments equipped with the Ploem epi-illumination (incident light fluorescent microscopy): thus weakly fluorescing antibodies could readily go undetected. In short, both the technical equipment and the tissue substrate to detect pancreatic ICA became simultaneously available at a time when the Immunology Laboratories in Edinburgh had painstakingly collected a bank of sera from insulin-dependent diabetics, with coexistent autoimmune endocrinopathy, which could be properly examined for evidence of pancreatic antibodies.

The studies presented here have indicated that islet-cell antibody is an immunoglobulin of IgG class, detectable by immunofluorescence, complement fixing and reacting with all cells in the islets. Later studies have confirmed that islet-cell antibodies are entirely of IgG/

IgG class but may be produced in much more restricted clonal responses (for example, IgG<sub>2</sub> subclass alone or IgG<sub>2</sub> + IgG<sub>4</sub>) than the widely polyclonal responses seen in gastric and thyroid antibodies; moreover, not all islet-cell antibodies are complement-fixing (Bottazzo et al, 1981). The antigen-antibody system was affected by various fixatives (table 2/3) in an identical fashion to the thyroid cytoplasmic and gastric parietal-cell cytoplasmic autoimmune systems. By analogy with these two systems, whose properties have been fully characterised, it seemed likely that the antigen(s) of the pancreatic islet-cell were also likely to be microsomal, i.e. comprised of membrane lipoproteins from subcellular fractions of smooth endoplasmic reticulum. Subsequent studies (Bottazzo et al, 1976; Lendrum et al, 1976; Doniach and Bottazzo, 1977) have strengthened this belief and have confirmed the organ-specificity of the antibody. By elegant studies with double immunofluorescence technique, using FITC-labelled antihuman IgG antibody and rhodamine-labelled anti-hormone antibodies, Bottazzo et al (1976) have demonstrated clearly that the antibody is not directed against islet hormones. The antibody is directed against cytoplasmic antigen, probably the cytoplasmic organelles concerned with hormone synthesis or secretion. This antigen is common to all four endocrine cell types in the islets of Langerhans (Bottazzo and Doniach, 1978) and is widely species-non-specific since human islet-cell antibody cross-reacts with islets from rat, guinea-pig and monkey.

The prevalence and significance of islet-cell antibody in diabetics and nondiabetics can now be considered. The initial studies presented here suggested that islet-cell antibody would be found in approximately one-third of insulin-dependent patients with overt autoimmune disease; in/

in about 20% of patients with circulating autoantibodies but without overt autoimmune disease; and in under 10% of diabetics who had no other clinical or serological expression of autoimmunity. Islet-cell antibody appeared to be overwhelmingly associated with insulin-dependent diabetes, although comparatively few insulin-independent diabetics had been tested. However, the presence of antibody in two insulin-independent diabetics, and in two nondiabetics with autoimmune disease, seemed to confirm that antibody was not related to prior treatment by insulin injections.

## 2/8 Results of further studies of pancreatic islet-cell cytoplasmic antibodies

Following the initial demonstration of pancreatic islet-cell cytoplasmic antibodies by Bottazzo et al (1974) and by the author, numerous further studies have been undertaken in Edinburgh, the Middlesex Hospital and a number of other laboratories. The most important and extensive of these studies are summarised in tables 2/5, 2/6 and 2/7, and now permit an accurate analysis of the prevalence and significance of this antibody. (The abbreviation 'ICA' is now applied to pancreatic islet-cell cytoplasmic antibody, to distinguish it from other islet-cell antibodies which have been subsequently described - see section 2/9).

Table 2/5 depicts the broad prevalence of ICA in several large studies which examined a total of nearly three thousand diabetics. It is immediately apparent that the highest overall prevalence of ICA is in insulin-dependent diabetics (IDDM) who suffer from at least one other coexistent autoimmune disease (usually thyrogastric or adrenal). In this type of diabetic population, having no regard to the duration of clinical diabetes, ICA prevalence is about 38% (Bottazzo et al, 1976; Irvine et al, 1977a). Even in the absence of overt autoimmune disease/



TABLE 2/5

The overall prevalence of pancreatic islet-cell antibody (ICA)  
in patients with insulin-dependent diabetes mellitus (IDDM),  
non-insulin-dependent diabetes (NIDDM), with and without  
associated autoimmune disease

Authors	Patients tested	Number Tested	Per cent Positive
Bottazzo et al (1976)	IDDM, overt autoimmune disease	120	38
Lendrum et al (1976a)	IDDM	829	38
	NIDDM	112	5.3
Irvine et al (1977a)	IDDM, overt autoimmune disease	60	38
	IDDM, thyrogastric autoantibodies	144	34.7
	IDDM, without autoimmune disease	588	22
	NIDDM, overt autoimmune disease	19	10
	NIDDM, without autoimmune disease	305	4.6
Del Prete et al (1977)	IDDM (onset aged under 35 years)	191	24.2
	IDDM (onset after age 35 years)	200	15.5
	NIDDM	373	8.3

\*Seven of these sixteen patients (44%) subsequently developed IDDM

TABLE 2/6

The correlation between the prevalence of ICA in insulin-dependent diabetics (IDDM) and the duration of clinically apparent diabetes

Authors	Patients tested	Number Tested	Per cent Positive
Christy et al (1976)	IDDM (mean duration 3.2 years)	38	55
Lendrum et al (1976a)	IDDM (duration one week)	20	85
	IDDM (duration one month)	54	50
	IDDM (duration above one year)	379	10-20
Muntefering et al (1976)	IDDM (duration less than 3 months)	40	70
Buschard et al (1976)	IDDM (onset under age 30 years)	40	70
Irvine et al (1977a)	IDDM (duration under one year)	135	59
	IDDM (duration 1 - 5 years)	143	30
	IDDM (duration above 5 years)	350	7.7
Del Prete et al (1977)	IDDM (duration less than 6 months)	22	45.4
	IDDM (duration 6 - 12 months)	24	29.1
	IDDM (duration 1 - 5 years)	52	19.2
	IDDM (duration above 5 years)	93	20.6
Ginsberg-Fellner et al (1982)	IDDM children (aged under 15 years, mean duration of DM 3.1 years)	74	65

TABLE 2/7

The prevalence of ICA in non-diabetic populations, with and without associated autoimmune disease, and in first degree relatives of IDDM children

Authors	Patients tested	Number Tested	Per cent Positive
MacGuish (this chapter)	'Healthy controls' (non diabetic, no autoimmune disease)	350	0
Bottazzo et al (1976)	Non diabetic, overt autoimmune disease	244	7.8
Irvine et al (1976, 1977a)	Non diabetic, overt autoimmune disease	522	} 3.8 <sup>a</sup>
	Non diabetic, suspected autoimmune disease	432	
	Non diabetic, first degree relatives of ICA-positive subjects	157	2.5 <sup>b</sup>
	'Healthy controls' (non diabetic, no autoimmune disease)	434	0.5 <sup>c</sup>
Del Prete et al (1977)	'Healthy controls' (normal GTT)	200	0
	'Non diabetic patients'	1159	1.7
Ginsberg-Fellner et al (1982)	First degree relatives of IDDM children	244	21
Rodger et al (1980)	Non diabetic adults (mean age 54)	3219	0.2
	Non diabetic children (age 12-18 yrs.)	537	0.4

GTT = oral glucose tolerance test

<sup>a</sup>Eight subjects were found to be diabetic by oral GTT

<sup>b</sup>One subject was found to be diabetic by oral GTT

<sup>c</sup>One subject was found to be diabetic by oral GTT

disease, the possession of circulating thyrogastric antibodies to IDDM implies that patients who exhibit this phenomenon will carry ICA in the same proportion as those with clinically apparent autoimmunity (Irvine et al, 1977a). By contrast, large populations of IDDM in whom the phenomenon of autoimmunity - clinical or serological - is specifically excluded will have an ICA prevalence of only about 20%. The exception is the prevalence of 38% in the large series described by Lendrum et al (1976a) but this population study included a large number of juveniles (57% were aged less than twenty years), a high percentage of patients with short duration of IDDM (50% had had diabetic symptoms for less than one year) and a significant proportion of patients with evidence of subclinical autoimmunity (23% had circulating thyroid and/or gastric parietal cell antibodies). All these factors are now known to increase the prevalence of ICA in an IDDM population.

Non-insulin-dependent diabetics (NIDDM) whether controlled by oral hypoglycaemics or by dietary measures alone, are very much less likely to possess ICA. The large series examined by Irvine et al (1977a) and by Lendrum et al (1976a) indicate an ICA prevalence of about 5% in such patients, although this figure doubles to about 10% if NIDDM with coexistent autoimmune diseases are considered separately (Irvine et al, 1977a). The rarity of ICA in NIDDM treated by diet alone is particularly striking: indeed Irvine et al found no ICA-positive subjects among one hundred and seven diet-treated patients whom they examined and if this subgroup of NIDDM is excluded from the analysis in table 2/5, the prevalence of ICA rises to 8% in a population of NIDDM treated with oral hypoglycaemic drugs (Irvine et al/



al, 1977a). Furthermore, ICA-positive diabetics treated with oral hypoglycaemics show a strong tendency to develop typical IDDM within three years from the detection of ICA (table 2/5), and thus it was postulated that the presence of ICA in such patients could be used as a 'marker' to identify patients with initially 'mild' diabetes who will go on to require insulin therapy (Irvine et al, 1977b). The figure of an ICA prevalence of 8% in NIDDM by Del Prete et al (1977) was obtained from a patient population in which one in five of the patients which they studied had a clinically overt, coexistent autoimmune disease and many were presumably taking oral hypoglycaemics. Del Prete and colleagues also examined a small number of patients with inherited insulin-independent diabetes appearing in early life - sometimes termed MODY (maturity-onset diabetes in youth, or Mason-type diabetes; Tattersall and Fajans, 1975) - and found no ICA-positive subject. This is an expected finding since MODY is a rare type of mild diabetes, inherited as a Mendelian dominant, not associated with autoimmunity or HLA type, and distinctly different from all other forms of diabetes (Bell et al, 1983).

These broad population studies are of considerable interest but are much enhanced when ICA prevalence is related to the duration of clinical diabetes. Table 2/6 summarises the results of some major studies in which duration of IDDM has been related to antibody prevalence. A strong correlation of ICA with duration of disease is apparent: up to 85% of IDDM will possess antibody if tested during the first week of clinical diabetes but thereafter the prevalence falls rapidly, and only about half of such patients will still be ICA-positive if they are tested after six to twelve months of IDDM. After one year of IDDM, antibody/

antibody prevalence has fallen to about 30% and after five years, ICA will only be detected in 10 - 20% of patients. Again it is noteworthy that the persistence of ICA-positivity with increasing duration of IDDM is related to the presence of coexistent autoimmune disease (AID): thus Irvine et al (1977a) detected ICA in about 60% of all IDDM tested within one year of diagnosis of diabetes but found after five years or more that ICA persisted in 26% of patients with coexistent overt AID and in only 7% of patients without AID. The series described by Christy et al (1976) and Ginsberg-Fellner et al (1982) are the exceptions to these broad general rules; however the latter workers specifically examined a population of IDDM children with remarkable personal and family backgrounds of autoimmune disease, and it seems probable that the series of Christy et al (where the patients' ages are not stated) also included a large number of similar juveniles. It is also possible, indeed probable, that differences in methodology contribute to the apparent discrepancies in results reported from different laboratories. For example, sera from the diabetics described by Ginsberg-Fellner et al (1982) were examined for the presence of ICA by using paraffin-embedded sections of pancreas from blood group O donors (Bouin's fixed human pancreas: Dobersen et al, 1979) rather than the snap-frozen sections of fresh human pancreas employed by most other workers. Differences in patient selection and methodology should not obscure the clear demonstration that first, ICA in IDDM disappear quickly from the sera of most patients without coexistent autoimmune disease and second, that ICA may persist for long periods in IDDM with coexistent autoimmune disease. The latter group of patients - with persistent ICA - behave like patients with a classical/

classical organ-specific autoimmune disease, such as Addison's disease (Irvine and Barnes, 1975), while the former - with transient ICA - are significantly different from patients with other autoimmune endocrinopathies.

The prevalence of ICA in NIDDM treated with oral hypoglycaemics is also influenced by duration of clinical diabetes, in a similar but less dramatic fashion to IDDM. Islet-cell antibodies were found in 20% of such patients at the time of diagnosis, the prevalence falling to about 10% after one to two years of clinical diabetes and to less than 10% after disease duration of more than two years (Irvine et al, 1977a). Again, persistence of ICA was found to correlate with other evidence of coexistent autoimmunity, either overt or serological (presence of thyrogastric antibodies). The tendency of NIDDM with ICA to progress to IDDM has already been noted (table 2/5) and was amply confirmed in a later study which demonstrated that 86% of such patients will progress from oral hypoglycaemic to insulin therapy within five years from diagnosis, as compared to 18% of ICA-negative patients (Irvine et al, 1979). Metabolic studies of NIDDM treated with oral hypoglycaemics have shown that ICA-positive patients have significantly higher blood glucose levels, and significantly lower insulin secretion when their response to a glucose load is compared to that of ICA-negative patients who are apparently clinically identical (Gray et al, 1980; Irvine et al, 1980b). In NIDDM treated by diet alone, the number of ICA-positive patients is vanishingly small and obviously is unaffected by duration of disease.

Table 2/7 describes the prevalence of ICA in several large series of nondiabetic individuals, with and without autoimmune disease. It can/

can be seen that the prevalence of ICA is exceedingly low in a putative normal population: the author found none in any of three hundred and fifty individuals and a similar finding was reported by Del Prete et al (1977) in a group of two hundred healthy individuals in whom oral glucose tolerance (GTT) was known to be normal. The large series of Irvine et al (1976) found only two ICA-positive subjects out of four hundred and thirty-four controls who were neither diabetic nor known to have endocrine disease, most being blood donors or healthy friends of hospital outpatients. Similar prevalence figures (0.2% in adults, 0.4% in children) were obtained when virtually the entire (nondiabetic) population of a small Australian town was tested for the presence of ICA (Rodger et al, 1980). However, when non-diabetics with autoimmune disease are considered, ICA prevalence in this type of population is between 4 - 8% (Irvine et al, 1976; Bottazzo et al, 1976), thus confirming and strengthening the association between antipancreatic autoimmunity and autoimmune disease affecting other organs. Thyroid disease and idiopathic Addison's disease, with or without coexistent polyendocrine autoimmune disease, are again the diseases most commonly associated with ICA-positivity. Of great interest was the observation that one quarter (eight out of thirty-one) of such patients identified by Irvine et al (1976) were diagnosed as diabetic by formal glucose tolerance testing, although none were previously known to be diabetic. This study provided the initial suggestion that ICA might be a 'marker' for asymptomatic or latent diabetes, or might play some part in the pathogenesis of beta cell failure. The same study searched for ICA among first-degree non-diabetic relatives of ICA-positive subjects (almost all of whom were diabetic/



diabetic) and found a prevalence of 2.5%; again one of those individuals was found to be diabetic on glucose tolerance testing (Irvine et al, 1976, 1977a). In a later investigation, carbohydrate tolerance in forty-two subjects with ICA but without clinical diabetes was followed for up to five years: within this time, nine had progressed to develop clinical diabetes and four had typical IDDM (Irvine, 1980).

The recent study of Ginsberg-Fellner et al (1982) is of particular importance since these authors found that more than one in five (21%) of first-degree (nondiabetic) relatives of diabetic children - i.e. the siblings and parents of these children - carried ICA in the sera. None of these individuals were frankly diabetic on oral glucose testing but many had significantly raised blood glucose levels following a glucose challenge and two (siblings who were both HLA-identical with their diabetic probands and who had been ICA-positive for three years and eighteen months respectively) developed typical IDDM during the study. This type of study - where an ostensibly normal individual is found to be ICA-positive, responds abnormally to a brief glucose challenge and then progresses to overt IDDM - can be compared with the prolonged challenge to carbohydrate tolerance that is imposed by normal pregnancy. Here again ICA is of value in predicting the progression to insulin-dependent diabetes: Steel and colleagues studied fifty 'normal' women with impaired glucose tolerance during pregnancy (defined as a capillary blood glucose above 6.6 mmols/l (200 mg/dl) two hours after a 50 g glucose load). Five women were found to have cytoplasmic ICA during pregnancy and three of these five developed typical IDDM within one year post-partum, a progression shown by none of the forty-five/

forty-five ICA-negative women (Steel et al, 1980). Further support for the belief that the presence of ICA in nondiabetics is a predictor for the eventual development of IDDM has come from a long prospective study of the pre-diabetic period in first-degree relatives of diabetic children. The authors searched for ICA in five hundred and twenty-eight first-degree nondiabetic relatives of one hundred and sixty children with IDDM: after two years of observation, six persons had developed IDDM and all were known to have circulating ICA for between four and thirty months before the onset of clinical diabetes (Gorsuch et al, 1981). All these studies have indicated the value of ICA in predating and predicting the eventual onset of IDDM, often after a lengthy period, in persons who have normal carbohydrate metabolism or minimal impairment of glucose tolerance when the antibody is first detected.

The studies in which the relationship of ICA to HLA typing has been examined can now be considered. The first such study was that of Lendrum et al (1976b), who found no association between the presence of ICA and any particular HLA phenotype. However these authors examined only one hundred and thirty-nine patients with IDDM, aged thirty years or under at the onset of disease, and detected ICA in only thirty-three cases. With such small numbers it is not possible to make a correlation between the HLA phenotype and the presence of ICA. By contrast Christy et al (1976) did find an association between the HLA antigen B8 and ICA in juvenile diabetics, although again only a small number of patients (thirty-eight cases) were examined. However the large series of Irvine et al (1977a) where HLA typing was performed in one hundred and twenty-two diabetics including one hundred ICA-positive/

ICA-positive subjects, found a clear association between HLA-B8, A1 and A1 +B8 and the presence of ICA. This series was also sufficiently large for the authors to correlate the presence of these HLA types with the presence of coexistent autoimmune disease and with the persistence of ICA for more than five years from the diagnosis of IDDM. Later studies have confirmed that IDDM with persistent ICA are HLA-B8 positive more often than antibody-negative patients (Bottazzo et al, 1978b; Cudworth, 1978; Irvine et al, 1978b; Cudworth et al, 1980). The importance of establishing an association between HLA-B8 and the presence and persistence of ICA in diabetes is illustrated by the observation of a very high prevalence of B8 in (Caucasian) individuals suffering from idiopathic (autoimmune) Addison's disease (Thomsen et al, 1975; Nerup et al, 1980; Irvine et al, 1980a), Grave's disease (Beck et al, 1977; Irvine et al, 1977c; Nerup et al, 1977; Cudworth et al, 1980), Hashimoto thyroiditis and primary hypothyroidism (Irvine et al, 1978a; Cudworth et al, 1980) - all regarded as classical organ-specific autoimmune diseases. Thus on the basis of islet-cell antibody and HLA studies alone, it would be reasonable to add IDDM with persistent ICA to the above group of diseases.

An association between IDDM (without regard to islet-cell antibodies) and HLA-B8 or B15 had been reported at about the same time in studies from both Denmark (Nerup et al, 1974b) and the U.K. (Cudworth and Woodrow, 1975, 1976) and the validity of an association between IDDM and certain HLA types has now been established beyond doubt (Cudworth et al, 1980; Nerup et al, 1980; Cudworth and Wolf, 1982). However it is important to note that the early studies of the relationships between HLA, IDDM and ICA were concerned only with the detection of HLA-A, -B and -C antigens/

antigens. These antigens, which are glycoproteins known to be present on virtually all nucleated body cells, are characterised on peripheral blood lymphocytes by using a wide range of antisera in a complement-dependent microlymphocytotoxicity test. In recent years more attention has been focused on the HLA-D and -DR (mean D-related) antigens in IDDM, the former being detected by mixed lymphocyte culture (MLC) techniques and the latter by serological methods. It is now clear that HLA-DW3-DR3 and DW4-DR4 represent the strongest and therefore the primary associations with IDDM; over 80% of young diabetics possess either DR3 or DR4 or both antigens. Associations with HLA-B, -C and -A antigens and complement factors are secondary, and are due to the phenomenon of linkage disequilibrium within the genes of the HLA system (Bodmer and Thompson, 1977; Schernthaner et al, 1977; Svejgaard et al, 1977; Nerup et al, 1980; Gudworth and Wolf, 1982). A fuller account of the relationship between HLA and diabetes is given in chapter seven.

In retrospect it can now be appreciated that the HLA-ICA studies alluded to above were examining secondary rather than primary associations between HLA and IDDM in Caucasian populations. If the HLA antigens coded for at all loci (HLA-A, -C, -B, -D and -DR) of chromosome 6 had been identified, an association would by now have been established between the presence of ICA and HLA-DW3-DR3-B8-A1 in IDDM. This is the first axis of HLA alleles which confers susceptibility to the development of insulin-dependent diabetes in Northern European Caucasians, probably through linkage with an immune response (Ir) gene (see chapter seven; figure 7/2). An analogy can again be drawn with Grave's disease and idiopathic Addison's disease, both of which are strongly DW3-B8 associated (Nerup et al, 1980). In fact, comparatively few studies/



studies have performed simultaneous HLA typing for D and DR antigens and a search for ICA in the same diabetic patients: the most recent is that of Ginsberg-Fellner et al (1982) (see also tables 2/6 and 2/7) who found HLA-DR3, -DR4 or both antigens in 81% of seventy-four children with IDDM and detected ICA in the sera of 64% of these patients. The sixty-four families studied by Ginsberg-Fellner et al were a racially heterogeneous group, including twenty-seven Asashkenazi-Jewish and fourteen Hispanic families as well as Southern Europeans and American Negroes: HLA-B8 has an extremely low prevalence in some of these ethnic groups so it is not surprising that an association between HLA-DR3-B8 and ICA was not established. However, it is of interest to note that these workers found a significantly reduced prevalence of ICA in first-degree relatives who carried the HLA-B7 antigen. The remarkably low frequency of HLA-B7 (and DR2, with which it is in linkage disequilibrium) in populations of IDDM has already led to the concept that the HLA-DR2-DW2-B7-A3 axis may itself be in linkage disequilibrium with an immune response (Ir) gene which provides a 'protective' function against beta cell damage (Nerup et al, 1980; Cudworth et al, 1980; Cudworth and Wolf, 1982; figure 7/2). If this is the case, individuals who possess HLA-DR2/B7 would indeed be expected to show less serological evidence of a disease process which could damage the endocrine pancreas.

In summary, following the mass of data and various studies described in this section, the following conclusions can be drawn regarding pancreatic cytoplasmic islet-cell antibody in diabetes mellitus:

- 1) ICA is found very commonly (in about 80% of patients) at the time of/

of diagnosis of overt IDDM. In most patients, the antibodies tend to disappear after a year or so of clinical disease but persistent ICA will be found in about 38% of patients who have concomitant endocrine autoimmune disease. Both the initial presence and the persistence of ICA in IDDM has been associated with the possession of HLA-B8 and A1, in Caucasian populations, and very probably with the possession of HLA-DR3-DW3. Absence of ICA is associated with possession of HLA-B7 and probably DR2.

2) The prevalence of ICA is much lower in patients with NIDDM, where there is no association with HLA. Antibody prevalence is particularly low in NIDDM treated by diet alone, probably no higher than in a nondiabetic population. In NIDDM treated with oral hypoglycaemics, ICA prevalence is about 10% in patients with coexistent autoimmune disease and about 5% in patients with no such associated disease. The detection of ICA is of value in NIDDM treated with oral drugs in predicting patients who will ultimately require insulin therapy: over 80% of antibody-positive patients may ultimately develop insulin-dependent diabetes, compared with only 18% of antibody-negative individuals (Irvine et al, 1979).

3) The prevalence of ICA in nondiabetic first-degree relatives of IDDM varies between 2.5% (Irvine et al, 1977a) and 21% (Ginsberg-Fellner et al, 1982), with much of the observed difference in prevalence being related to the methodology employed for antibody detection or the testing of nondiabetics with established autoimmune disease. The presence of ICA in relatives of an insulin-dependent diabetic may also identify individuals who already have impaired beta cell function (Irvine et al, 1976; Ginsberg-Fellner et al, 1982) and who may progress to/

to develop IDDM (Irvine, 1980; Bottazzo et al, 1981; Gorsuch et al, 1981).

4) The prevalence of ICA in a background (nondiabetic) or putative 'normal' population is very low indeed, probably well below 1%, in persons who have no clinical or serological evidence of autoimmune disease. In a population of nondiabetics with autoimmune disease, ICA prevalence lies between 4% and 8%. The presence of ICA in such cases may again be of predictive value insofar as diabetes can be demonstrated in about one-quarter of these asymptomatic individuals by formal glucose tolerance testing.

5) Multiple studies have now demonstrated the value of cytoplasmic ICA as a marker for the eventual development of clinical diabetes mellitus. Moreover, there is a strong tendency for such patients to progress, with time, through a clinical spectrum of increasingly severe insulin deficiency, such that dietary carbohydrate restriction, oral hypoglycaemic drugs and eventually exogenous insulin injections will be required. There are many clinical situations - for example, predicting diabetes in the sibling of an IDDM proband or forecasting a failure of sulphonylurea therapy in a drug-treated NIDDM - where such a marker would be a valuable aid to patient care. The detection of cytoplasmic ICA in an asymptomatic, normal individual should always be treated seriously and the rarity of ICA in a background population adds to its value as a marker for diabetes; it is incomparably better than HLA typing, where calculation demonstrates that less than 1% of DR3- or DR4-positive healthy subjects (in the absence of a strong family history) are likely to develop IDDM (Cudworth and Wolf, 1982). If a standardised, reproducible assay for ICA could be developed - perhaps/

perhaps a radioimmunoassay - detection of these antibodies need no longer be confined to specialised immunology laboratories. ICA assays could then be available in district general hospitals and would be used by clinicians responsible for diabetic care. An analogy can be drawn with the present status of human glycosylated protein assays, which were a research tool until recently but are now used on a wide scale to assess diabetic control.

6) A pathogenic role for ICA in the process of beta cell destruction - as opposed to its value as a marker for latent or eventual diabetes - has not yet been established. Almost certainly it plays no direct role, since the cytoplasmic antigen(s) against which it is directed are found in all cell types in the islets of Langerhans (Bottazzo and Doniach, 1978) and IDDM is characterised by highly selective destruction of insulin-producing beta cells. The other endocrine cell populations in the islets - glucagon-producing A cells, somatostatin-producing D cells, pancreatic polypeptide-producing PP cells - survive apparently unharmed in IDDM, even after long duration of disease (Orci et al, 1976; Gepts and De Mey, 1978; Gepts and LeCompte, 1981). Furthermore, by classical immunological theory, a model involving recognition (and subsequent destruction) of beta cells by the immune system would require target molecules or antigens to reside in the beta cell membrane, facing the external environment (Roitt, 1980; McConnell et al, 1981). Cytoplasmic ICA is directed against an intracellular antigen, albeit unknown, which should be inaccessible to the antibody while the beta cell remains viable. Appreciation of these practical and theoretical difficulties in assigning a pathogenic role for cytoplasmic ICA has provided a stimulus to search for other antibodies/



antibodies directed against components of the endocrine pancreas; the results of these searches will now be briefly described.

2/9 The detection of other islet-cell antibodies in diabetes mellitus

In recent years, pancreatic islet-cell antibodies have been detected in several different assay systems: these antibodies, and the methods used for their detection, are summarised in table 2/8. Shortly after the initial detection of cytoplasmic antibodies (ICA), various workers reported the detection of antibodies which would only bind to antigen(s) presented on the cell exterior: these antibodies are now collectively referred to as islet-cell surface antibodies (ICSA). The first study to describe ICSA was that of MacLaren et al (1975), who reported the presence of ICSA in 87% of sera from thirty-eight children with recent onset IDDM, using immunofluorescence on an insulinoma cell line established in 1959. This study is suspect, as the insulinoma cells did not produce insulin (MacLaren, 1977), and unfortunately the cell line is now extinct. The results could not be reproduced with other insulinomas. In 1978 Lernmark et al used a similar technique on dispersed rat islet cells in suspension, and found positive results in twenty-two out of eighty-eight (32%) of cases of IDDM in children, compared to 4% of sera from healthy controls. Not all the islet cells in their preparations showed surface fluorescence with positive sera, but no attempt was made to prove that the staining was confined to beta cells (Lernmark et al, 1978). The use of human islet cells in an assay system to detect ICSA was described by Pujol-Borrell et al (1982), who cultured pancreases from 11 - 24 week old human fetuses and demonstrated surface binding immunoglobulins in/

TABLE 2/8Pancreatic islet cell antibodies detected by various assay systems

Antibody	Method of Detection	Authors
Islet cell cytoplasmic antibody (ICA)	Immunofluorescence on	Bottazzo et al (1974)
	tissue sections	MacGuish et al (1974)
	(group 0 human pancreas)	Dobersen et al (1979)
Complement-fixing islet cell antibodies	Immunofluorescence on	Betterle et al (1980)
	tissue sections (group 0 human pancreas)	Bottazzo et al (1980)
Islet cell surface antibody (ICSA)	Immunofluorescence of	
	islet cell suspensions	
	Insulinoma cell line:	MacLaren et al (1975)
	Rodent islet cells:	Lernmark et al (1978)
	Human foetal pancreas:	Pujol-Borrell et al (1982)
Complement-dependent cytotoxic islet cell antibodies	$^{51}\text{Cr}$ release from damaged	Soderstrum et al (1979)
	islet cells or vital	Dobersen et al (1980)
	staining (ethidium bromide)	Idahl et al (1980)
Antibody-dependent cellular cytotoxic antibodies	$^{51}\text{Cr}$ release from lysed	Lernmark et al (1981b)
	rodent islet cells	Soderstrum and Lernmark (1982)
Immunoprecipitating islet cell antibodies	Polyacrylamide gel	Lernmark and
	electrophoresis of	Baekkeskov (1981)
	precipitated islet cell	Baekkeskov et al
	antigens	(1981, 1982)

in sera from nine out of eleven newly-diagnosed insulin-dependent diabetics. By using a four-layer double immunofluorescence technique with anti-hormone sera these workers also demonstrated that the surface antibody was certainly staining insulin-secreting cells, but owing to rarity of A and D cells in the foetal cultures they were not able to exclude the possibility that the antibody also reacted with glucagon or somatostatin cells (Pujol-Borrell et al, 1982). However, a quantitative analysis of cell surface immunofluorescence in a flow cytofluorophotometer (a fluorescence-activated cell sorter) has disclosed that antibodies in IDD sera with ICSA bind preferentially to the pancreatic beta cells (Van de Winkel et al, 1981). Thus it seems likely that ICSA are more specifically directed against the beta cell than cytoplasmic ICA.

The rodent islet-cell assay for ICSA described by Lernmark et al has now been applied, in similar fashion to the earlier studies of cytoplasmic ICA, to examine populations of IDDM patients and to compare the prevalence of ICSA with that of ICA. It is now established that ICSA is present in about 80% of newly-diagnosed IDD patients and in 2 to 4% of controls (Lernmark et al, 1978, 1981a). In one study of one hundred and forty-four patients with IDDM, seventy-three (51%) were found to have islet-cell antibodies detected by cytoplasmic and/or cell surface assays. Cytoplasmic ICA alone were detected in thirty-seven patients (25%), ICA + ICSA in twenty-three patients (16%) and ICA alone in thirteen patients (9%) (Freedman et al, 1979). A later prospective study by the same group searched for ICA and ICSA in sera obtained from thirty-three children and adolescents immediately after the diagnosis of IDDM. On the day of diagnosis, before the institution of/

of insulin therapy, thirty out of thirty-three (91%) were antibody-positive, 67% having ICA and 67% having ICSA, although both antibodies did not always coexist in the same patient. Antibody prevalence was followed prospectively in seventeen patients and fell to 57% within nine months of clinical diabetes. These results and others involving cytotoxic islet-cell antibodies (Dobersen et al, 1980) have indicated that first, the prevalence of ICA and ICSA is roughly parallel in IDDM; second, that not all patients with islet-cell antibodies necessarily have both cytoplasmic and surface antibodies simultaneously; and third, that ICA and ICSA both appear as transient phenomena in the majority of newly-diagnosed diabetics and tend to disappear through the passage of time.

As with cytoplasmic ICA, the pathogenetic role of ICSA has still to be defined. In one in vitro experiment, ICSA was found to inhibit the biosynthesis but not the secretion of insulin by rodent islets when incubated with immunoglobulin from IDD serum containing ICSA (Lernmark et al, 1979). It remains to be shown whether the antigen recognised by ICSA is a protein, perhaps a membrane receptor for agents regulating beta cell function.

Two other immunological phenomena which involve antibodies bound to living cells are of possible importance in destruction of beta cells and have been applied to the study of insulin-dependent diabetes. First, cell surface-bound antibodies are known to be recognised by components of the complement systems. Recognition of IgM or IgG molecules on the cell surface, with binding of complement, results in activation of the complement cascade reaction which leads to a proteolytic attack on the cell/



cell membrane. The complement-induced membrane lesions result in an increased plasma membrane permeability and eventually to cell death. Fresh serum (Idahl et al, 1980) and serum from IDDM patients (Soderstrum et al, 1979; Dobersen et al, 1980, 1981), or immunoglobulins obtained from such sera, appear to be capable of mediating a complement-dependent cytotoxic reaction against a variety of islet cell preparations. The assay reactions for these complement-dependent cytotoxic islet-cell antibodies (table 2/8) utilised dispersed islet cells (prepared from rodent pancreas or human insulinoma) which are first labelled with radioactive chromium and then incubated with serum samples. After washing by centrifugation the cells are incubated with animal (guinea-pig) serum as a source of complement. The release of radioactive chromium is taken as an index of cell viability, while lysed cells are identified by their capacity to take up ethidium bromide. Proof that the lysed cells are beta cells is obtained by staining the preparation with fluorescein-conjugated antibody to insulin. Dobersen et al have already used this elegant technique to demonstrate that complement dependent islet-cell cytotoxic antibodies correlate with ICSA but not ICA in diabetic sera; that about 80% of cells lysed in the reaction are beta cells; and that the mere presence of these cytotoxic antibodies does not seem sufficient to produce diabetes since the antibodies can be demonstrated in about one quarter of sera from (nondiabetic) first-degree relatives of IDDM probands (Dobersen et al, 1980, 1981). This type of islet-cell antibody assay system is of great potential interest and importance since it provides evidence of a capacity for islet-cell antibodies to damage beta cells and, since the assay employs radioisotope release, it offers the promise of an assay system which is both/

both objective and capable of being standardised.

Second, cell surface antibodies may be the target of cytotoxic lymphocytes, the so-called natural killer cells (K cells). The K cell can be found among the circulating monocytes (West et al, 1978) and is thought to possess several surface receptors which bind the constant portion of the antibody molecule. It appears that the major proportion of circulating K cells can be identified by their ability to form rosettes when incubated at low temperatures with high concentrations of sheep red blood cells (E): thus they are referred to as low affinity E-rosette forming cells (E-RFC) (West et al, 1978). The K cell acts either as an armed cell, with antibodies attached, or attacks cells with an antibody bound to the surface; the resulting reaction is known as antibody-dependent cellular cytotoxicity (table 2/8). Already there is evidence that increased levels of low affinity E-RFC are found in IDDM (Sensi et al, 1982) and preliminary reports (in an animal model, using xenogenic rat islet cell surface antiserum against rodent islet cells) have suggested that ICSA have the capacity to mediate this type of cytotoxic reaction (Lernmark et al, 1981b; Soderstrum and Lernmark, 1982). The role of human ICSA in this type of reaction has yet to be established.

A further recently described assay system has been used to demonstrate the presence of immunoprecipitating islet cell antibodies in sera from IDDM (Lernmark and Baekkeskov, 1981; Baekkeskov et al, 1981, 1982). Human pancreatic islets were isolated from cadaver kidney donors, labelled biosynthetically with  $^{35}\text{S}$ -methionine and solubilised in detergents (NP-40). The solubilised radioactive-labelled islet cell proteins were then incubated with sera from newly-diagnosed/

newly-diagnosed diabetic children and normal controls. Immune complexes were isolated and analysed by gel electrophoresis; the analysis demonstrated that sera from newly-diagnosed diabetics contained antibodies against at least two proteins (molecular weight approximately 64,000 and 38,000 daltons) present in normal human islets. In contrast, sera from normal controls and from IDDM with disease duration of more than one year did not contain antibodies against such proteins (Lernmark and Baekkeskov, 1981; Baekkeskov et al, 1982). The proteins identified in these interesting experiments might be the antigen(s) involved in studies which demonstrated the presence of circulating soluble immune complexes in the sera of forty-five out of one hundred and ten newly-diagnosed diabetics (41%) and 53% of patients with established IDDM (Irvine et al, 1977d, 1980c) and it may be anticipated that further studies with modern techniques will lead to more precise localisation and characterisation of the target antigens in this antigen-antibody system.

In conclusion, the numerous and complex studies described and reviewed in this chapter have discussed the identification of a variety of islet-cell antibodies in human diabetes by several different immunological assay systems. The list is not exhaustive: for example, ICSA can also be detected by a radioactive immunoglobulin-specific reagent such as  $^{125}\text{I}$ -protein A in a competition assay (radioligand assay) (Lernmark et al, 1980); ICA have been detected by immunocytochemical staining with peroxidase-conjugated antibodies; ICA can be detected by immunofluorescent complement-fixation tests (Betterle et al, 1980; Bottazzo et al, 1980); and there is a little evidence to suggest that hypothetical islet-cell receptor antibodies may also exist (Lernmark et/

et al, 1979). A survey of the present status and possible future significance of islet-cell antibodies must emphasise the following points:

- 1) The explosion of interest in the immunology of diabetes mellitus over the past twelve years has led to the identification of several different antibodies directed against various components of the islets of Langerhans. Some antibodies - notably cytoplasmic ICA and (to a lesser extent) islet cell surface antibody - have already been rigorously studied in diabetic populations. Islet-cell antibodies have been found in the majority of insulin-dependent diabetics at the time when the clinical disease first appears, have been demonstrated to be transient phenomena in a majority of patients and have been shown to persist for long periods of time in diabetics with coexistent autoimmune disease. The presence of islet-cell antibodies has also been demonstrated to predate the onset of insulin-dependent diabetes, to predict an eventual need for insulin therapy in mild diabetes and to identify asymptomatic individuals who are either normal or have trivial impairment of glucose tolerance but eventually progress to clinical diabetes.
- 2) There is a clamant need to refine the precision and the reproducibility of assay systems used to detect islet-cell antibodies. This is particularly true of assay systems using immunofluorescence as the detection method, since multicentre studies have indicated concordant scores of only 50 to 60% for IDD sera examined by four different laboratories (Drash, 1981). Some of these difficulties can be resolved by standardisation of the tissue substrates and the microscopic equipment used in the assays; the present need to use human cadaveric/



cadaveric material is likely to disappear as defined suspensions of endocrine islet cells (Eisenbarth et al, 1981a) and eventually continuous, cloned specific endocrine cell lines (Gazdav et al, 1980) become available. Those assay systems which employ radioisotope release are particularly attractive since the end result is objective, and should permit quantitative and reproducible antibody assays to be established. Such assays would be a valuable adjunct to the clinical management of carbohydrate intolerance.

3) The mechanism whereby any of the islet-cell antibodies might exert a pathogenetic influence on beta cell destruction is presently obscure, as is the interrelationship between different antibodies in the same individual. Many of these difficulties might be resolved if the nature and site of the antigens against which they are directed was known. The recent advent of human monoclonal autoantibody production - by the fusion of human myeloma cells with antibody-producing B cells (Eisenbarth et al, 1981b) - is an exciting development with a number of practical implications. For example, a radioimmunoassay for islet-cell antibody could be developed by using the labelled monoclonal autoantibody as a tracer. Furthermore, it might be possible to produce a form of immune intervention with antibodies raised against the variable regions of the monoclonal autoantibody (anti-idiotypic antibodies). Moreover, passive transfer of monoclonal antibodies (Eisenbarth et al, 1981c) to immunosuppressed rodents could be used to test the hypothesis that a particular islet-cell antibody was diabetogenic. Finally, since diabetic sera have been shown to immunoprecipitate some human islet-cell proteins, it should be possible to extract the messenger RNA for these proteins and determine their structure by molecular cloning.

This/

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This type of approach might eventually allow those islet-cell antigens to be made by polypeptide synthesis (Lerner et al, 1981). All these new techniques of immunology and molecular biology can be used to further the study of abnormal immune mechanisms in the genesis of diabetes mellitus.

4) The detection of islet-cell antibodies has given powerful support to the whole concept of an autoimmune form of diabetes, especially when considered in conjunction for the evidence (chapter three) for cell-mediated antipancreatic autoimmunity in this disease. Even with the limitations of the present techniques, it is clearly important to examine both humoral and cellular autoimmune phenomena in the same patients. Some preliminary studies of this nature undertaken by the author are considered in chapter four.

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Part of this work has already been published (MacGuish et al, 1974a).

## CHAPTER THREE

## STUDIES OF CELL-MEDIATED AUTOIMMUNITY IN DIABETES

3/1 Introduction and purpose of studies

The leucocyte migration technique (LMT), as developed by Bendixen and Soborg (1969) for studies of immune phenomena in human disease, has been used by several investigators to provide in vitro evidence of cellular (delayed type) hypersensitivity to specific antigens in diabetes mellitus. The theoretical basis of the test depends on the ability of lymphocytes from a sensitised individual, when cultured in contact with specific antigen, to release a variety of soluble factors known collectively as lymphokines (Dumonde, 1970): among these substances is migration inhibition factor (MIF), which interferes with or inhibits the normal in vitro migration of white blood cells in culture medium. In the context of autoimmune disease, the LMT has been widely used to provide presumptive evidence of cell-mediated immunity to thyroglobulin and thyroid tissue antigens in Hashimoto thyroiditis (Soborg and Halberg, 1968; Brostoff, 1970; Calder and Irvine, 1975), intrinsic factor and gastric tissue antigens in pernicious anaemia (Brostoff, 1970; Finlayson et al, 1971; Goldstone et al, 1973), adrenal antigen in Addison's disease (Nerup et al, 1969) and liver antigen in primary biliary cirrhosis (Brostoff, 1970; Smith et al, 1972).

The first successful demonstration of antipancreatic cellular hypersensitivity in diabetes by this technique was accomplished by Nerup et al (1971), who prepared an antigen of very high islet-cell content from porcine pancreas in which atrophy of exocrine tissue had been induced by surgical ligation of the pancreatic duct for eight weeks before the organ was removed. The antigenic material was obtained/

obtained by homogenising the atrophic pancreas and separating the homogenate by differential centrifugation: the fraction obtained with ultracentrifugation (104000 g) was used as the antigen. This preparation induced inhibition of leucocyte migration in peripheral-blood samples from fifteen of twenty-two diabetics, all but four of whom were insulin-dependent and most aged under forty-five years. Intracutaneous injection of the same preparation into six diabetics with positive in vitro reactions (inhibition) induced typical delayed-type hypersensitivity in four, thus strengthening the validity of the results as an indicator of in vivo cell-mediated immunity. It is particularly important to note that migration inhibition was detected by Nerup et al in five patients who had received no antidiabetic therapy of any description at the time of study, and in whom there was therefore no question of prior sensitisation by exogenous insulin injection. Likewise no correlation was found between the presence of insulin antibodies and a positive IMT in the insulin-treated diabetics. In the light of these findings, it seems reasonable to regard this study as demonstrating a phenomenon of true autoimmunity against the endocrine pancreas in diabetes.

The position regarding migration inhibition with antigens from other organs is more controversial and has centred round the question as to whether liver mitochondria are also capable of inhibiting leucocyte migration in diabetes. In the study described above, Nerup et al cultured diabetic leucocytes in the presence of antigens prepared from porcine liver and kidney, as well as porcine and bovine insulin, and found no migration inhibition in diabetics to any of these antigens. In contrast Richens et al (1973) used antigens consisting of the mitochondrial/



mitochondrial fraction of human and rat liver homogenates, and described striking migration inhibition of leucocytes from twenty-six of thirty-five insulin-dependent diabetics (mean age 27 years) and four of twelve insulin-independent diabetics (mean age 62 years). Reactivity to liver mitochondria could of course be considered as a general nonspecific 'marker' for autoimmune disease, since inhibition of leucocyte migration is commonly induced by this antigen in patients with Hashimoto thyroiditis (Calder et al, 1972) and pernicious anaemia (Goldstone et al, 1973). Thus the findings of Richens et al, if confirmed, would provide further evidence for aligning diabetes mellitus with this group of diseases but might question the organ-specificity of the reaction observed by Nerup et al.

In the light of these published papers, which were the only studies extant when the work described below commenced, it was considered that further investigation in this aspect of immunology and diabetes should be made to assess the effect of a pancreatic antigen derived from human rather than animal source; to examine the response of cells from non-insulin-dependent patients in the same assay system; and to attempt to clarify the reasons for the differing results of Nerup et al and Richens et al.

The studies described below were therefore undertaken with the following specific objectives:

1. To examine the effects of an antigen prepared from human pancreas on the in vitro migration of leucocytes from diabetics.
2. To test the response to this antigen in each of the major types of clinical diabetes (insulin-dependent, non-insulin-dependent) rather than confining the tests to insulin-dependent patients.

3. To observe the effects of liver mitochondrial antigens on leucocyte migration in diabetes.
4. To determine whether soluble antigens of pancreatic hormones (insulin, glucagon) had any effect on leucocyte migration in diabetes.

### 3/2 Migration inhibition studies in diabetes

#### Patients used for study

Samples from one hundred and one diabetics and fifty-six non-diabetic controls were examined for their response to various antigens in the IMT. These subjects were divided into the following groups:

Young diabetics. These were thirty-one insulin-dependent diabetics (eighteen women and thirteen men), aged from eighteen to forty-two years (mean 28.5), and insulin-treated for between one and nineteen years (mean 7.4).

Young controls. These were twenty-seven healthy nondiabetic volunteers who were selected to allow statistical comparisons with the young diabetics. Fifteen were women and twelve men, aged from eighteen to forty-two years (mean 28.6).

Older diabetics on oral hypoglycaemic agents (OHA). These were thirty-four diabetics diagnosed at age forty years or more who were controlled by dietary carbohydrate restriction plus a sulphonylurea. Twelve were women and fourteen men, aged from forty-five to seventy years (mean 59.7).

Older diet-treated diabetics. These thirty-six patients were controlled by simple carbohydrate restriction only; twenty-one were women and fifteen men, aged from forty-six to sixty-nine years (mean 60.5).

Older controls. Twenty-three healthy nondiabetic volunteers were/

were studied, selected by sex (thirteen women and ten men) and age (range forty-eight to sixty-nine, mean 57.4 years) to allow valid statistical comparison with the previous two groups.

Positive reactors. This small group, included to check the potency of the mitochondrial preparation, consisted of one patient with primary biliary cirrhosis and five with Hashimoto thyroiditis, known to show inhibition of leucocyte migration when previously tested with mitochondria (Calder et al, 1972).

All the diabetics regularly attended the Diabetic Department of Edinburgh Royal Infirmary and when blood was taken for study, were well-controlled and free of infection. Control subjects were healthy volunteers, with the exception of the 'positive reactors'.

#### Antigens used in study

Human pancreas was obtained immediately after death from a previously healthy young man who sustained fatal head injuries in a car accident and whose kidneys were used for transplant; this organ was made available through the kindness of Mr. J.B. Morton, Lecturer in Surgery, University of Edinburgh. The fresh pancreas was cut into small pieces, homogenised in sterile phosphate-buffered saline (Appendix I/1), filtered through fine gauze and then centrifuged at 700 g for twenty minutes. The supernatant was used as antigen, its protein content being adjusted to 10 mg/ml and stored at  $-20^{\circ}\text{C}$  in small aliquots. When required these were thawed and further diluted with tissue culture fluid consisting of Eagle's Basal Medium (EBM, Wellcome Reagents Ltd.: Appendix I/1), supplemented with 10 per cent foetal calf serum. Pilot experiments showed the highest nontoxic concentration of antigen to be 200  $\mu\text{g/ml}$  which was/

was used for all experiments.

Mitochondrial preparations were obtained from the livers of young Wistar rats by the differential centrifugation technique of Nerup and Bendixen (1969), this antigen being used at a protein concentration of 200  $\mu\text{g/ml}$  culture medium. A similar preparation was made from human liver using an identical technique and this human mitochondrial preparation was used at a concentration of 50  $\mu\text{g/ml}$  culture medium.

Purified bovine insulin was obtained from the Lilly Research Laboratories, Indianapolis (lot No. 615-D63-5), through the kindness of Dr. J.A. Galloway. This insulin had been repeatedly recrystallised to remove the a-component (which consists of high molecular weight non-hormonal pancreatic proteins) with which commercial insulin is contaminated. The crystalline insulin was diluted in culture medium to give a working concentration of 116  $\mu\text{g/ml}$  culture medium.

Purified bovine glucagon was obtained from the same source and used at a concentration of 100  $\mu\text{g/ml}$  culture medium.

#### Leucocyte migration test (IMT)

The IMT was performed by the method of Bendixen and Soborg (1969), with the minor modifications incorporated by Calder et al (1972). Fifty ml venous blood was collected from patients and controls, heparinised (preservative-free heparin, 10 units/ml) and allowed to sediment at 37°C for one to two hours. The leucocyte-rich plasma was removed, centrifuged at 150g for ten minutes and the cell pellet washed three times in ERM. Contaminating red cells were lysed with ammonium chloride (0.83 per cent) for five minutes, and the resultant leucocyte pellet washed a further three times with ERM. The washed cells were then/



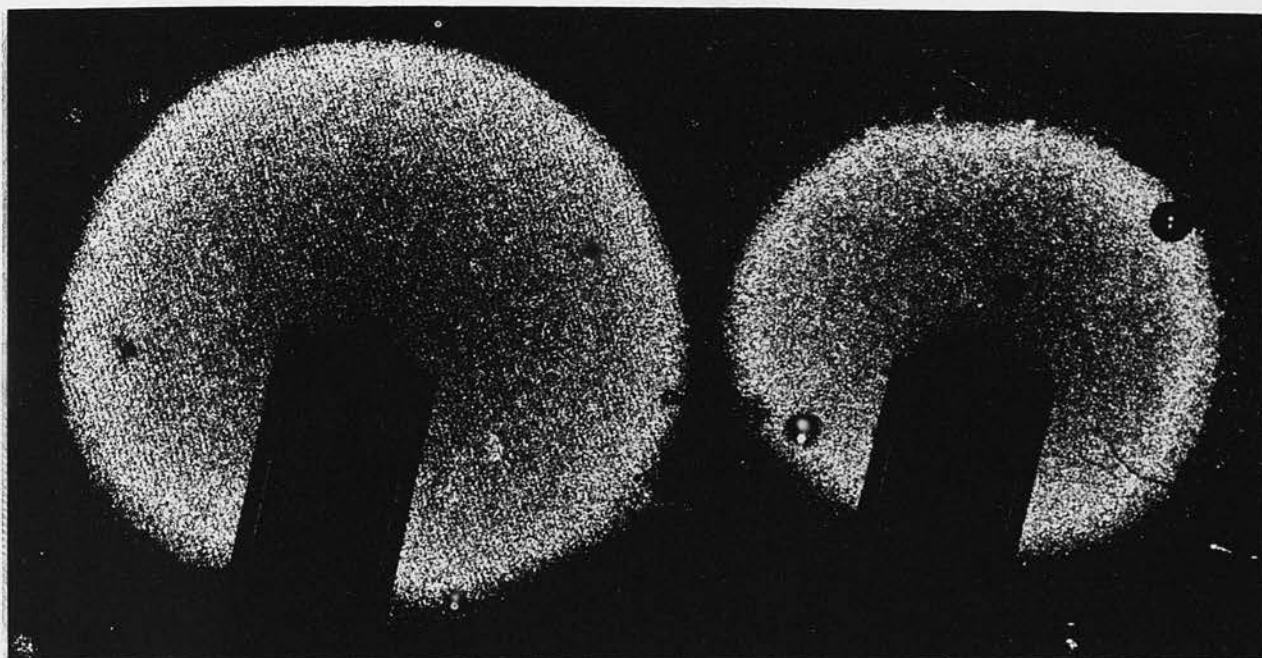
then resuspended in EBM with 10 per cent foetal calf serum. Capillary tubes (25  $\mu$ l) were filled with the cell suspension, sealed at one end and centrifuged at 150g for five minutes. The tubes were then cut 1 mm below the cell-fluid interface and the cell pellet positioned, with a dab of silicone grease, in a leucocyte migration chamber (obtained from Sterilin Ltd.). One series of at least three chambers was filled with culture medium alone, and a second series with culture medium plus antigen. The chambers were sealed with glass coverslips and incubated on a flat surface at 37°C for twenty-four hours. The fanlike pattern of migration was then projected (Projectina microscope) and the area measured by planimetry. The effect of antigen on cell migration (the 'migration index') was expressed as a percentage of migration without antigen using the formula:

$$\text{Migration index} = \frac{\text{Mean migration with antigen}}{\text{Mean migration without antigen}} \times 100\%$$

A figure of less than 80 per cent was taken to indicate significant inhibition of migration and above 120 per cent, significant stimulation; this range is identical to that adopted by other investigators (Nerup et al, 1971; Richens et al, 1973) and represents two standard deviations above and below the mean migration index shown by six normal subjects to pancreatic antigen during the pilot experiments. An example of inhibition of leucocyte migration in the presence of antigen is shown in figure 3/1.

### Results

The results of leucocyte migration tests in diabetics and controls were compared statistically by the Wilcoxon rank sum test (Wilcoxon, 1947/

FIGURE 3/1

Inhibition of leucocyte migration in the presence of pancreatic antigen. On the left, leucocytes have migrated normally from the capillary tube into culture medium; on the right, migration has been inhibited by the addition of pancreatic antigen to the culture medium.

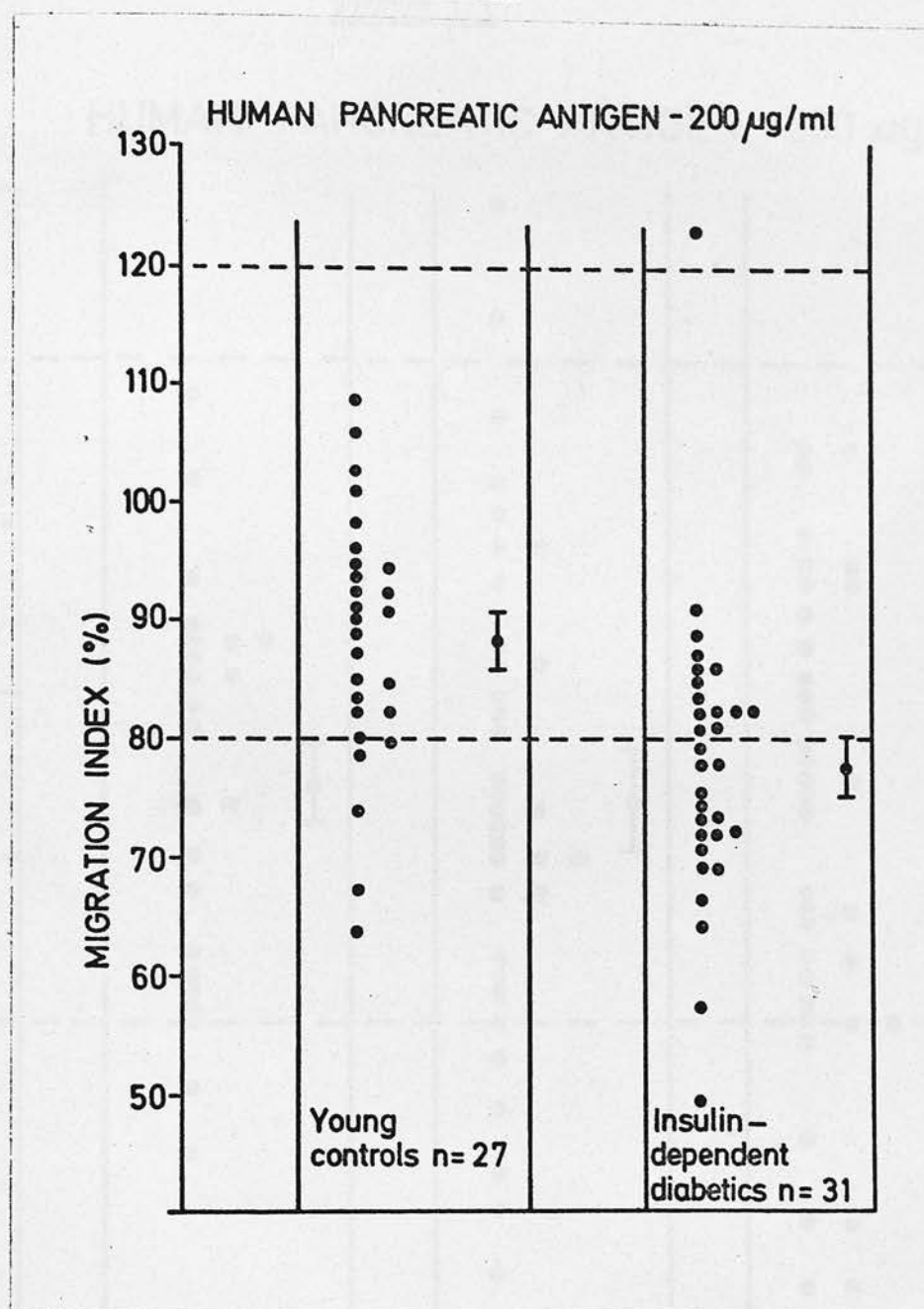
From MacCuish et al (1974b)

1947). All diabetics and normal controls were tested with human pancreatic antigen (figures 3/2, 3/3). Seventeen of the thirty-one young diabetics showed inhibition as compared with only four of the twenty-seven young controls; the mean migration index for the former ( $77.7 \pm 2.3$  S.E.M.) was significantly lower than in the latter ( $88.3 \pm 2.1$ ). In contrast only six older diabetics on OHA and six of those on diet alone showed migration inhibition compared with two of the corresponding controls: the respective mean migration indices were  $93.4 \pm 2.8$ ,  $91.5 \pm 2.4$  and  $94.4 \pm 2.5$  and do not differ significantly. The young insulin-dependent diabetics differed significantly from all other groups, both individually and collectively ( $p < 0.01$  by Wilcoxon's test) but there were otherwise no significant differences between the groups (table 3/1).

The results of tests using rat liver mitochondrial antigen are shown in figure 3/4. The mean migration indices  $\pm$  S.E.M. for young diabetics, normal controls and older diabetics were  $88.5 \pm 3.2$ ,  $92.7 \pm 2.3$  and  $91.9 \pm 2.3$  respectively, these groups not differing significantly. In contrast all patients with Hashimoto thyroiditis or primary biliary cirrhosis, included as positive reactors, showed marked migration inhibition (mean  $69.5 \pm 2.4$ ), confirming the potency of mitochondrial antigen: this group differed significantly from all other groups, both collectively and individually ( $p < 0.01$  by Wilcoxon's test, table 3/2).

Very similar results were obtained using human instead of rat liver mitochondria. The mean migration indices with this antigen in young diabetics, normal controls and older diabetics were  $93.5 \pm 2.9$ ,  $94.8 \pm 2.5$  and  $94.1 \pm 2.4$  respectively, these groups not differing significantly/

FIGURE 3/2

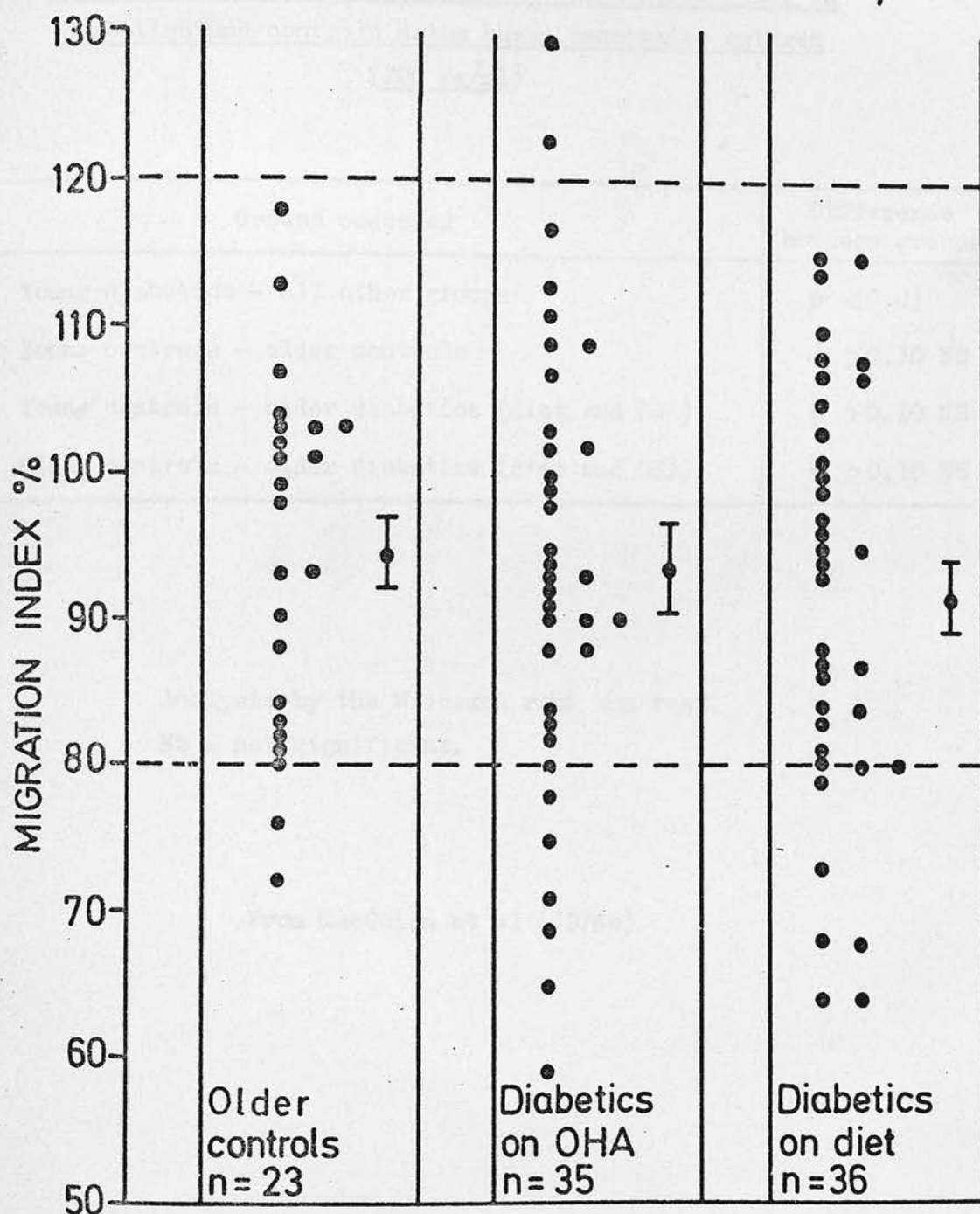


Leucocyte migration tests with human pancreatic antigen in young controls and insulin-dependent diabetics. Inhibition of migration is shown by seventeen (55%) of the diabetics as compared with four (15%) controls.

From MacGuish et al (1974b)



FIGURE 3/3

HUMAN PANCREATIC ANTIGEN - 200  $\mu\text{g/ml}$ 

Leucocyte migration tests with human pancreatic antigen in older controls and insulin-independent diabetics. Inhibition of migration is shown by six (17%) each of diabetics on OHA and diet respectively, compared with two (9%) of the controls.

TABLE 3/1

Statistical comparisons of leucocyte migration tests in  
diabetics and controls using human pancreatic antigen  
(200  $\mu$ g/ml)

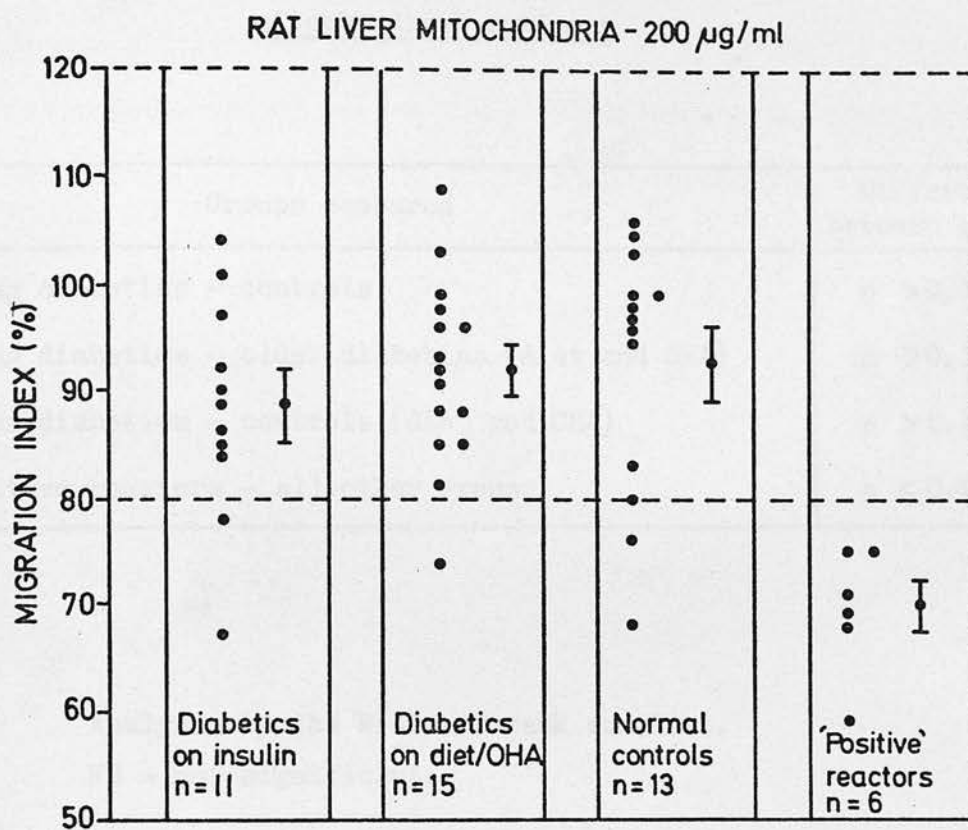
Groups compared	Difference between groups
Young diabetics - all other groups	p <0.01
Young controls - older controls	p >0.10 NS
Young controls - older diabetics (diet and OHA)	p >0.10 NS
Older controls - older diabetics (diet and OHA)	p >0.10 NS

Analysis by the Wilcoxon rank sum test.

NS = not significant.

From MacCuish et al (1974b)

FIGURE 3/4



Leucocyte migration tests with rat liver mitochondrial antigen in insulin-dependent and independent diabetics, normal controls and 'positive reactors' (see text). Only the group of positive reactors shows significant inhibition of migration.

From MacGuish et al (1974b)

TABLE 3/2

Statistical comparisons of leucocyte migration tests in  
diabetics and controls using rat liver and human liver  
mitochondrial antigens

Groups compared	Difference between groups
Young diabetics - controls	p > 0.10 NS
Young diabetics - older diabetics (diet and OHA)	p > 0.10 NS
Older diabetics - controls (diet and OHA)	p > 0.10 NS
Positive reactors - all other groups	p < 0.01

Analysis by the Wilcoxon rank sum test.

NS = not significant.

From MacCuish et al (1974b)



significantly. This potency of this human antigen preparation was again confirmed by the small group of positive reactors, all of whom demonstrated marked migration inhibition (mean  $66.2 \pm 2.8$ ) and differed significantly from all other groups (table 3/2).

Bovine insulin was used as antigen to test leucocyte migration in all the young insulin-treated diabetics and corresponding normal controls; none showed migration inhibition to insulin at a concentration of  $116 \mu\text{g/ml}$  culture medium (equivalent to  $3.2 \text{ i.u./ml}$  culture medium). Above this level a toxic effect was observed. The mean migration index was virtually identical in diabetics and controls ( $94.8 \pm 4.1$  and  $93.9 \pm 3.7$  respectively).

Bovine glucagon was likewise without effect on leucocyte migration in the young diabetics and corresponding controls. The mean migration index to this antigen (at a concentration of  $100 \mu\text{g/ml}$  culture medium) in diabetics and controls was  $98.1 \pm 3.6$  and  $100.2 \pm 4.0$  respectively.

### 3/3 Discussion of migration inhibition studies in diabetes

Two further studies of antigen-induced migration inhibition in diabetes were reported while the present work was in progress and the results obtained by all the investigators who have used this test are summarised in table 3/3. As stated, Nerup et al (1971) first described inhibition of leucocyte migration to a pancreatic antigen in fifteen of twenty-two diabetics, all but four of whom were insulin-dependent and most aged under forty-five years. The antigen used was derived from pooled porcine pancreas in which atrophy of exocrine tissue had been induced by prolonged ligation of the pancreatic duct. The same investigators (Nerup et al, 1973, 1974; table 3/3) later used an antigen/

TABLE 3/3

Summary of migration inhibition studies in diabetes mellitus,  
using pancreatic and nonpancreatic antigens

Authors	Antigens used in the LMT		Comments
	Positive Results (inhibition)	Negative Results (no inhibition)	
Nerup et al (1971)	Porcine pancreas (islets)	Pig liver mitochondria.  Porcine kidney.  Pig and bovine insulin.	Inhibition in 65% of 22 diabetics (most young, insulin-dependent).
Nerup et al (1973, 1974)	Foetal calf pancreas (homogenate)	Foetal calf liver.  Foetal calf thymus.	Inhibition in 28% of 112 diabetics (all types).
Richens et al (1973)	Rat liver mitochondria.  Human liver mitochondria.	Rat kidney mitochondria.  Rat adrenal mitochondria.	Inhibition in 63% of 47 diabetics. Only work showing inhibition to mitochondria.
MacCuish et al (1974b)	Human pancreas (homogenate)	Rat liver mitochondria.  Human liver mitochondria.  Bovine insulin and glucagon.	Inhibition in 29% of 101 diabetics (all types)*
Richens (1974)	Human insulinoma.  Rat pancreas.	-	Unpublished (Personal communication)
This thesis (chapter 4)	Human insulinoma.	-	Inhibition in 50% of 28 diabetics (juvenile-onset)

LMT = Leucocyte migration test.

\* = Studies described in this chapter.

antigen of homogenated foetal calf pancreas to demonstrate migration inhibition in thirty-one (28%) of one hundred and twelve diabetics. Inhibition was shown in both juvenile- and maturity-onset diabetics, occurring in insulin-dependent and insulin-independent patients; however, the phenomenon was most commonly found in young recently-diagnosed diabetics, irrespective of therapy.

The present studies using human pancreas have demonstrated migration inhibition in a comparable number of diabetics (29% of one hundred and one patients) and confirm that cell-mediated immunity, as judged by the LMT, is found most often in insulin-dependent patients with juvenile-onset diabetes. The most obvious deficiencies of this investigation - i.e. the use of a relatively crude homogenate of whole human pancreas, and the failure to test juvenile-onset diabetics who were either untreated or insulin-independent - have been remedied by the later studies using an antigen of human islet-cell tumour (table 3/3; chapter four of this thesis). The results of the present and previous studies would therefore seem to indicate the existence in diabetes of a state of cell-mediated immunity to antigen(s) which is present in the pancreas and species-non-specific (demonstrable with porcine, bovine and human pancreas). The fact that migration inhibition is found in untreated and insulin-independent subjects provides the main basis for claiming that the reaction reflects a state of true antipancreatic cellular autoimmunity, rather than a spurious hypersensitivity resulting from exogenous insulin injections. The validity of this in vitro test as an indicator of equivalent in vivo antipancreatic hypersensitivity would seem to be confirmed by the finding by Nerup et al that diabetics with a positive reaction to the/

the LMT will also show typical delayed-type hypersensitivity (i.e. a type IV reaction) if the same antigen is injected subcutaneously. No in vivo tests have been performed in the present investigation, in view of the ethical difficulty inherent in injecting healthy volunteers with antigens of human tissue.

The position regarding migration inhibition in diabetics to antigens from other organs remains controversial. Richens et al (1973) showed inhibition in twenty-six of thirty-five young insulin-dependent diabetics and in four of twelve elderly insulin-independent diabetics tested with an antigen consisting of liver mitochondria from young Sprague-Dawley; subsequent studies by the same investigators have suggested that the antigenic component is localised to the inner mitochondrial membrane (Richens et al, 1974). Richens et al also described migration inhibition in a small number of insulin-dependent patients tested with human liver mitochondria but found no inhibition to antigens of mitochondria from rat kidney and adrenal. On the other hand, Nerup et al (1971, 1973, 1974) used antigens of porcine liver and kidney, foetal calf liver and thymus in their series of diabetics and did not demonstrate migration inhibition to any of these antigens. The results of the present investigation, which has used human and rat liver mitochondrial preparations of proven potency, are in agreement with the latter investigators. The reasons for the differences between the various studies are not clear but may in part reflect different methods of antigen preparation: Richens et al used the method of Zamecnik and Keller (1954) while all the other studies have used the method of Nerup and Bendixen (1969), in which the tissue is centrifuged at different speeds and in different buffers. Until this/



this question is resolved, it is not possible to state with certainty whether or not the general reactivity to mitochondria that is a feature of thyrogastric autoimmune disease is also shown in diabetes.

Further studies are also needed to define which component of pancreatic tissue acts as antigen towards the lymphocyte population in diabetic patients. On the one hand the highest number of positive results with the LMT has been obtained by using antigens of highest islet-cell content (Nerup et al, 1971; this thesis, chapter four), suggesting that the antigen is located in the endocrine part of the pancreas. On the other hand, the use of purified hormones extracted from islet-cell tissue - insulin and glucagon - has failed to inhibit migration of leucocytes in the LMT. This observation might therefore suggest that the antigenic material is non-hormonal (or at least, not the fraction of islet-cell polypeptides that to date has been tested for antigenic effects), or might merely reflect the technical limitations of the LMT, which works well with particulate antigens (i.e. fractions of solid tissue) but rarely with soluble antigens. The possibility that pancreatic hormones might show some antigenic activity in diabetes if used in an alternative in vitro test system for cell-mediated hypersensitivity has been explored in the studies described below.

### 3/4 Antigen-induced lymphocyte transformation studies in diabetes

The transformation of cultured small lymphocytes (presumably T cells) to lymphoblasts in the presence of antigen is another widely-used in vitro test of cell-mediated immune function (Bloom, 1971; Rocklin, 1974). As with studies using a mitogen such as phytohaemagglutinin (PHA), the effect of antigen is assessed by direct examination of/

of cultured cell morphology using the light microscope, or more conveniently by pulsing the cultures with a radioactive DNA precursor (e.g.  $^3\text{H}$ -thymidine) which will be incorporated into the nuclei of transforming cells: the extent of blastogenesis can subsequently be measured by beta counting of the extracted nucleoprotein.

In the context of autoimmune disease, the transformation technique has been used to demonstrate the presence of lymphoid cells sensitised to intrinsic factor in patients with pernicious anaemia (Tai and McGuigan, 1969) and to thyroglobulin in patients with Hashimoto thyroiditis (Ehrenfeld et al, 1971). Human diabetes would seem to be an equally suitable model for the transformation technique in an attempt to demonstrate the existence of lymphoid cell subpopulations which 'recognise' components or secretions of the endocrine pancreas. However there is little information concerning antigen-induced lymphocyte transformation in this disease except in the context of insulin allergy, where various investigators (Halpern et al, 1967; Federlin et al, 1968, 1971) have shown that bovine insulin can induce blastogenesis in lymphocytes with both immediate (urticarial) and delayed type (cutaneous) insulin allergy. The present investigation was therefore undertaken to examine the question of cell-mediated hypersensitivity to insulin in Type 1 diabetics who had no such in vivo evidence of insulin allergy; in addition, a small number of untreated diabetics were also tested to determine whether these patients showed any evidence of hypersensitivity before treatment with exogenous insulin. The latter investigation was considered to be of particular importance in the context of autoimmunity and in view of the failure of insulin hypersensitivity to be demonstrable by the IMT. When it became/

became apparent that lymphocyte transformation in diabetes could indeed be induced by insulin, the isolated A and B chains of insulin were further examined in an attempt to localise the immunogenic portion of the insulin molecule.

#### Patients used for study

Sixty-five diabetics were studied. Forty-five were women and twenty were men, aged from sixteen to seventy-one years (mean 42.1). Fifty were established diabetics and had been insulin-treated for between six months and twenty-four years. Fifteen were newly-diagnosed diabetics, five of whom had been insulin-treated for three weeks or less and ten of whom were untreated at the time of study. All were attending the Diabetic Outpatient Clinic, Edinburgh Royal Infirmary, and in all (except for the newly-diagnosed) the disorder was well-controlled as judged by the following criteria: steady weight and insulin dosage; absence of thirst or polyuria; no heavy glycosuria; no ketonuria; mid-morning blood glucose of less than 14 mmol/l (250 mg/dl). All were taking insulin of bovine origin, prepared either as soluble (Regular) insulin or in a sustained-release form (Protamine-Zinc or IZS Lente insulin). None had evidence of immediate or delayed-type insulin allergy.

The control group comprised thirty healthy nondiabetic volunteers. For statistical purposes it was selected to be comparable in terms of sex (twenty women, ten men) and age (range fifteen to seventy-two, mean 40.8 years) with the diabetic group. Most controls were laboratory or hospital personnel; a few were hospital outpatients who were not known to have endocrine or immunological disease.

#### Antigens/

### Antigens

Bovine and porcine insulins, highly purified and of known potency and composition (table 3/4), were obtained from the Lilly Research Laboratories through the courtesy of Dr. J.A. Galloway. Bovine insulin A chain and B chain (aminoethylated), and porcine insulin A chain (S-sulphonate) were obtained from the same source. All preparations were in crystalline powder form. On the day of use they were dissolved in a mixture of 10% sterile phosphate buffered saline/80% Eagle's Basal Medium/10% foetal calf serum.

The optimal concentration of insulin for use in these investigations was determined by measuring the blastogenic effect of insulin on lymphocytes from a diabetic with known insulin allergy; the patient was a twenty-two year old man who had a daily insulin requirement in excess of 200 units in addition to cutaneous reactions to bovine insulin injections, and who therefore showed evidence of both humoral and cell-mediated insulin hypersensitivity. Lymphocytes were obtained from this patient on two separate occasions, prepared and cultured as described below, and exposed to varying concentrations of bovine insulin antigen. The results (table 3/5) show that maximal blastogenesis was stimulated with insulin at a concentration of 500  $\mu\text{g/ml}$  (10  $\mu\text{g/culture}$ ), no significant increase in lymphocyte  $^3\text{H}$ -thymidine uptake being induced by higher concentrations of insulin. Thus on the basis of this preliminary dose-response analysis, all experiments were performed with solutions containing 500  $\mu\text{g/ml}$  intact insulin or insulin fragment.

### Sample collection and lymphocyte culture

Fifteen ml venous blood was removed from each patient and control at/



TABLE 3/4

Lymphocyte transformation in diabetics: composition of  
Lilly bovine and porcine insulins used as antigens

Content *	Bovine Insulin	Porcine Insulin
Proinsulin	0.06	0.15
Glucagon	0.007	0.002
Zinc	0.80	0.70
Monodesamido insulin	13.0	7.0
'Arginine' insulins	1.0	2.0
Potency (U/mg)	25.4	25.4

\* per cent

From MacGuish et al (1975)

TABLE 3/5

Ability of varying concentrations of bovine insulin to induce transformation (judged by  $^3\text{H}$ -thymidine uptake) of cultured lymphocytes from a diabetic with insulin allergy

	Control*	Cultures plus Bovine Insulin Antigen*			
		50 $\mu\text{g}$ /ml	500 $\mu\text{g}$ /ml	1000 $\mu\text{g}$ /ml	5000 $\mu\text{g}$ /ml
		1 $\mu\text{g}$ /culture	10 $\mu\text{g}$ /culture	20 $\mu\text{g}$ /culture	100 $\mu\text{g}$ /culture
First Assay	1,080	8,720	12,480	13,640	10,390
Second Assay	1,769	9,550	14,570	14,210	12,620

\* All results as cpm.

No significant increase of blastogenesis is obtained by adding more than 10  $\mu\text{g}$  insulin per culture

From MacGuish et al (1975)

at mid-morning and anticoagulated with preservative-free heparin. Lymphocytes were prepared from whole blood by the methods (density centrifugation, repeated washing in EEM) described in chapter five of this thesis, and suspended in EEM with 10% foetal calf serum at a concentration of  $1 \times 10^6$  cells/ml.

A portion of the lymphocytes from each subject was cultured in the presence of the mitogen phytohaemagglutinin (PHA) to provide a convenient assessment of the adequacy of general cell-mediated immune function. The lymphocyte transformation response to PHA was measured by exactly the same method as described in chapter five of this thesis: a mitogen dose-response curve for each subject could therefore be constructed by using PHA at three different concentrations. The radioactivity of stimulated cells was determined as before by an automatic beta counter and results are expressed as counts per minute (cpm).

The remaining lymphocytes from each patient and control were cultured in the presence of insulin antigen. The technique used was modified from the PHA micromethod and is briefly summarised as follows: aliquots of  $2 \times 10^5$  lymphocytes were pipetted into the wells of plastic tissue culture plates (Cooke Microtiter). All experiments were performed in triplicate and consisted of a control row of cultures without antigen, and rows to which 20  $\mu$ l of the required solution (i.e. 10  $\mu$ g insulin or insulin fragment) was added. The plates were gassed with a 95% air/5% CO<sub>2</sub> atmosphere and incubated at 37°C in sealed, humidified containers. After five days the cultures were labelled with <sup>3</sup>H-thymidine (specific activity 5Ci/mmol, dose 0.5  $\mu$ ci/culture) and terminated sixteen hours later by harvesting the cells onto glassfibre filter/

filter discs and preparing them for scintillation counting. The prepared samples were counted in an automatic beta counter.

Results were expressed as counts per minute (cpm); the effect of antigen in stimulating blastogenesis (the 'transformation index') was expressed by the formula:

$$\text{Transformation Index} = \frac{\text{cpm of cultures with antigen}}{\text{cpm of cultures without antigen}}$$

The normal range of transformation indices using intact insulin antigens was obtained from the mean transformation index  $\pm$  2 standard deviations of the results given by both insulins in the control group (i.e. 60 experiments). The range in normals by this method was found to be 0.57 - 1.45 (mean  $0.01 \pm 0.44$ ) and a transformation index above 1.45 was thus taken to indicate significant antigenic stimulation of blastogenesis. Several cultures from controls and diabetics were examined by light microscopy to confirm that the uptake of radioactive label was paralleled by morphological blast transformation.

All diabetics and controls were tested with intact bovine and porcine insulin antigens. Lymphocytes from those patients who showed significant transformation were further tested with bovine A and B chain and porcine A chain.

## Results

### Mitogenic effects of PHA

The maximal PHA response of diabetics and controls (i.e. the highest lymphocyte incorporation of  $^3\text{H}$ -thymidine label induced by one of the three concentrations of PHA used) is shown in tables 3/6 - 3/8. The mean maximal PHA response in the thirty controls was  $23.1 \pm 4.3$  (expressed/



TABLE 3/6

Ability of bovine insulin, porcine insulin and PHA to induce transformation (judged by  $^3\text{H}$ -thymidine uptake) of cultured lymphocytes from thirty normal subjects

Age/sex	Control cpm	Bovine Insulin		Porcine Insulin		Maximal PHA Response cpm
		cpm	T.I.	cpm	T.I.	
15/m	939	546	0.58	751	0.80	25,100
72/f	1,318	806	0.61	1,216	0.92	17,900
29/f	704	501	0.71	613	0.87	44,400
19/m	1,188	893	0.75	1,463	1.23	22,800
34/f	1,026	784	0.76	1,281	1.24	15,000
34/f	823	669	0.80	707	0.86	16,300
66/f	975	792	0.81	800	0.82	8,700
32/f	1,133	941	0.83	1,497	1.32	22,500
53/m	1,572	1,322	0.85	1,352	0.86	38,400
24/f	759	654	0.86	502	0.66	16,800
58/f	1,033	914	0.88	1,109	1.07	11,100
36/m	1,007	899	0.89	1,025	1.02	14,900
26/f	659	589	0.90	594	0.90	26,100
68/f	1,583	1,521	0.96	1,456	0.92	21,200
32/m	1,292	1,250	0.97	1,066	0.83	10,800
48/f	875	860	0.98	684	0.78	19,800
41/f	958	940	0.98	937	0.98	19,200
28/f	1,106	1,104	1.00	1,175	1.06	44,700
36/f	1,136	1,178	1.04	1,264	1.11	14,100
18/m	848	891	1.05	903	1.06	29,300
69/m	710	750	1.05	726	1.02	22,500
54/f	1,285	1,363	1.06	926	0.72	7,600
26/f	955	1,042	1.09	1,781	1.86	20,300
46/f	797	880	1.10	1,260	1.58	27,400
48/f	923	1,017	1.10	991	1.07	13,300
45/m	1,714	1,910	1.11	1,980	1.16	23,500
32/f	1,073	1,222	1.14	1,300	1.21	38,500
57/m	1,809	2,583	1.43	1,835	1.01	16,700
36/m	1,465	2,109	1.44	1,584	1.08	30,000
48/f	968	1,457	1.51	1,327	1.37	54,100

Results are expressed both as counts per minute (cpm) and transformation index (T.I. - see text). All figures means of triplicate cultures.

Ability of bovine insulin, porcine insulin and PHA to induce transformation (judged by  $^3\text{H}$ -thymidine uptake) of cultured lymphocytes from fifty established insulin-dependent diabetics

Study No.	Age/Sex	Control cpm	Bovine Insulin		Porcine Insulin		Maximal PHA Response cpm
			cpm	T.I.	cpm	T.I.	
1	27/m	964	705	0.73	1,039	1.08	35,900
2	50/f	1,541	1,123	0.73	1,326	0.86	17,500
3	43/f	1,495	1,146	0.85	1,053	0.80	22,400
4	40/f	809	728	0.90	802	0.98	18,700
5	31/m	1,475	1,388	0.94	1,400	0.95	26,400
6	62/f	878	843	0.96	805	0.92	24,100
7	65/f	1,096	1,071	0.98	956	0.87	30,700
8	40/f	1,167	1,172	1.00	1,006	0.86	17,600
9	59/f	1,122	1,120	1.00	1,327	1.10	20,400
10	66/f	994	1,001	1.01	1,351	1.36	18,500
11	45/m	1,226	1,270	1.04	1,164	0.95	26,400
12	38/f	915	959	1.05	1,049	1.15	25,000
13	42/m	1,518	1,597	1.05	2,173	1.43	13,900
14	25/f	728	760	1.05	881	1.21	17,400
15	48/f	1,306	1,427	1.09	1,259	0.96	7,800
16	46/f	811	900	1.11	853	1.05	41,800
17	55/f	963	1,079	1.12	1,014	1.05	21,400
18	20/f	870	979	1.13	1,357	1.56	33,300
19	37/f	931	1,051	1.13	863	0.93	27,600
20	69/m	1,071	1,210	1.13	1,089	1.02	10,600
21	23/f	1,000	1,140	1.14	1,320	1.32	19,300
22	46/m	939	1,067	1.14	1,221	1.30	27,400
23	41/f	1,043	1,210	1.16	1,079	1.03	12,700
24	45/f	720	848	1.18	794	1.10	15,500
25	21/f	923	1,155	1.25	1,609	1.74	50,600
26	70/f	1,320	1,690	1.28	1,990	1.51	20,400
27	25/f	1,448	1,853	1.28	1,445	1.00	26,600
28	62/m	962	1,228	1.28	1,062	1.11	38,200
29	14/f	634	818	1.29	799	1.26	8,500
30	27/f	859	1,112	1.30	1,120	1.30	11,900
31	22/m	951	1,109	1.31	1,561	1.83	21,100
32	67/f	869	1,169	1.34	927	1.07	21,200
33	57/m	1,446	1,952	1.35	2,053	1.42	39,500
34	52/f	1,094	1,557	1.42	1,503	1.37	14,000
35	46/m	832	1,192	1.43	1,278	1.53	10,300
36	42/f	1,391	2,130	1.53	2,465	1.77	18,200
37	30/f	989	1,533	1.55	1,553	1.57	35,900
38	41/f	943	1,525	1.62	1,719	1.82	19,200
39	37/f	885	1,430	1.62	1,686	1.90	27,900
40	48/f	1,000	1,659	1.66	1,809	1.81	26,700
41	61/f	1,832	3,077	1.68	2,994	1.63	18,900
42	54/f	1,016	1,802	1.77	1,641	1.61	20,500
43	59/f	1,547	2,769	1.79	2,614	1.69	14,100
44	52/f	843	1,512	1.79	2,366	2.80	17,600
45	61/m	1,042	1,963	1.88	1,777	1.71	26,000
46	62/f	897	1,790	1.99	1,282	1.42	47,100
47	26/f	734	1,508	2.06	1,454	1.98	21,300
48	71/f	1,458	3,164	2.17	3,762	2.58	41,000
49	38/m	864	2,449	2.83	2,049	2.38	13,900
50	39/f	1,046	3,797	3.63	4,707	4.50	34,500

TABLE 3/8

Ability of bovine insulin, porcine insulin and PHA to induce transformation (judged by  $^3\text{H}$ -thymidine uptake) of cultured lymphocytes from fifteen newly-diagnosed diabetics

Study No.	Age/Sex	Control cpm	Bovine Insulin		Porcine Insulin		Maximal PHA Response cpm
			cpm	T.I.	cpm	T.I.	
51	58/f	1,505	1,472	0.98	1,430	0.95	17,700
52	37/m	841	832	0.99	699	0.83	7,800
53	19/m	1,043	1,029	0.99	1,343	1.29	19,400
54	42/m	891	972	1.09	881	0.99	9,900
55	18/m	619	680	1.10	930	1.50	13,800
56	19/f	1,473	2,458	1.67	2,591	1.76	38,000
57	23/f	757	1,493	1.98	1,520	2.00	25,400
58	30/m	802	1,626	2.03	1,729	2.16	16,500
59	16/m	1,138	2,675	2.35	2,281	2.00	25,400
60	19/f	1,307	5,313	4.07	5,925	4.53	16,600
61	21/f	795	1,089	1.37	1,026	1.29	22,400
62	24/m	680	850	1.25	911	1.34	20,900
63	17/f	1,070	1,947	1.82	1,530	1.43	9,900
64	29/m	923	1,228	1.33	1,476	1.60	19,700
65	37/f	856	813	0.95	899	0.05	23,600

Results as in Table 3/6

Modified from MacGuish et al (1975)



(expressed as  $\text{cpm} \times 10^3 \pm \text{S.E.M.}$ ) while the corresponding figure in sixty-five diabetics was  $21.9 \pm 4.8$ . The two groups do not differ significantly in PHA responsiveness and the results confirm the earlier observation (MacCuish et al, 1974c; this thesis, chapter five) that mitogen-induced lymphocyte transformation is normal in diabetes mellitus, except at times of severe metabolic decompensation.

The maximal PHA response in five control subjects was further measured in lymphocyte cultures to which 10  $\mu\text{g}$  bovine or porcine insulin had been added. The results (table 3/9) indicate that neither insulin has an appreciable effect on  $^3\text{H}$ -thymidine uptake in the presence of PHA when added to forty-eight-hour cultures in the stated concentrations.

#### Antigenic effects of intact insulins

The effects of bovine and porcine insulin antigens in inducing blastogenesis of lymphocytes from controls and diabetics are presented in tables 3/6 - 3/8 (as absolute cpm) and in figures 3/5 and 3/6. Only one normal subject showed significant transformation using bovine insulin antigen; in contrast fifteen established and six newly-diagnosed diabetics (four untreated, two insulin-treated for less than three weeks) showed significant transformation, their indices ranging from 1.53 to 4.07 (figure 3/5). The groups of both established and newly-diagnosed diabetics differed significantly ( $p < 0.01$  by Wilcoxon's test) from the control group.

Experiments using porcine insulin as antigen (figure 3/6) gave a virtually identical pattern. Two control subjects showed significant transformation, as compared with twenty established and seven newly-diagnosed diabetics (four untreated, three treated for less/



TABLE 3/9

Effects of bovine and porcine insulin on PHA-induced transformation of lymphocytes from five normal subjects

Age/Sex	Maximal $^3\text{H}$ -thymidine uptake (cpm)		
	With PHA	PHA + B.I.*	PHA + P.I.*
36/m	32,000	35,000	31,000
19/m	25,000	24,000	22,000
32/m	9,000	8,000	9,000
26/f	28,000	26,000	26,000
36/m	14,000	16,000	14,000

Neither insulin has a significant effect on the  $^3\text{H}$ -thymidine uptake of PHA-stimulated lymphocytes.

\* B.I. - Bovine insulin 10  $\mu\text{g}$ /culture

P.I. - Porcine insulin 10  $\mu\text{g}$ /culture

From MacCuish et al (1975)

FIGURE 3/5 (OPPOSITE):

Lymphocyte transformation in controls and diabetics by bovine insulin antigen. The mean transformation index  $\pm$  S.E.M. is shown to the right of the individual results for each group. Dotted lines (---) indicate the normal range (mean  $\pm$  2 S.D.) of transformation indices in control subjects. Significant transformation (index 1.45) is shown by fifteen established and six newly-diagnosed diabetics as compared to one control. Both diabetic groups differ significantly ( $p < 0.01$ ) from the control group.

Modified from MacGuish et al (1975)

FIGURE 3/5

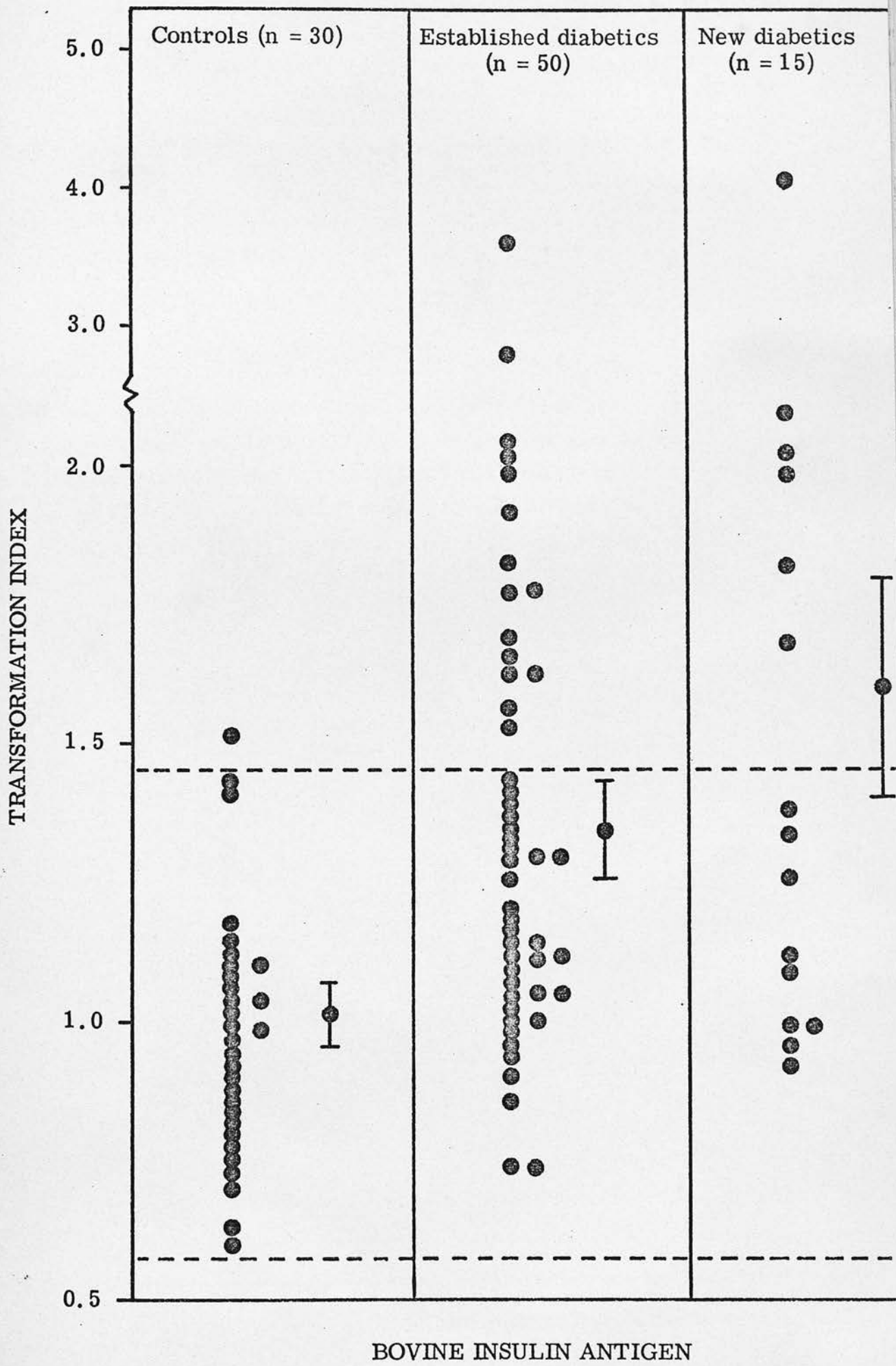


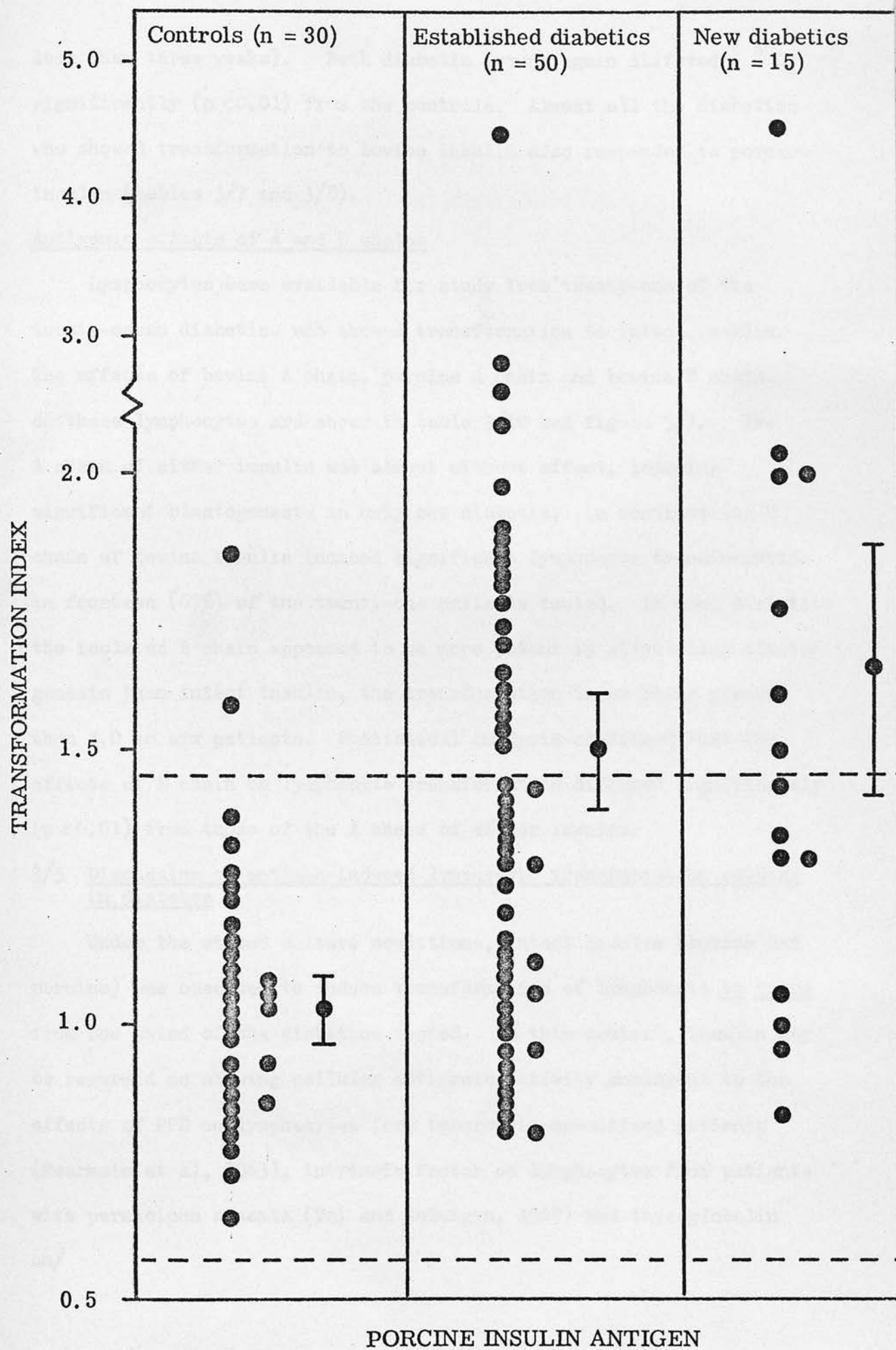
FIGURE 3/6 (OPPOSITE):

Lymphocyte transformation in controls and diabetics induced by porcine insulin antigen. Symbols as in figure 3/5. Significant transformation is shown by twenty established and seven newly-diagnosed diabetics as compared to two controls. Both diabetic groups differ significantly ( $p < 0.01$ ) from the control group.

Modified from MacGuish et al (1975)



FIGURE 3/6



less than three weeks). Both diabetic groups again differed significantly ( $p < 0.01$ ) from the controls. Almost all the diabetics who showed transformation to bovine insulin also responded to porcine insulin (tables 3/7 and 3/8).

#### Antigenic effects of A and B chains

Lymphocytes were available for study from twenty-one of the twenty-seven diabetics who showed transformation to intact insulin. The effects of bovine A chain, porcine A chain and bovine B chain on these lymphocytes are shown in table 3/10 and figure 3/7. The A chain of either insulin was almost without effect, inducing significant blastogenesis in only one diabetic; in contrast the B chain of bovine insulin induced significant lymphocyte transformation in fourteen (67%) of the twenty-one patients tested. In some diabetics the isolated B chain appeared to be more potent in stimulating blastogenesis than intact insulin, the transformation index being greater than 4.0 in six patients. Statistical analysis confirmed that the effects of B chain on lymphocyte transformation differed significantly ( $p < 0.01$ ) from those of the A chain of either insulin.

#### 3/5 Discussion of antigen-induced lymphocyte transformation studies in diabetes

Under the stated culture conditions, intact insulin (bovine and porcine) was observed to induce transformation of lymphocyte in vitro from one third of the diabetics tested. In this context, insulin may be regarded as showing cellular antigenic activity analogous to the effects of PPD on lymphocytes from tuberculin-sensitised patients (Pearmain et al, 1963), intrinsic factor on lymphocytes from patients with pernicious anaemia (Tai and McGuigan, 1969) and thyroglobulin on/

TABLE 3/10

Ability of bovine insulin A chain, porcine insulin A chain and bovine B chain to induce transformation (judged by  $^3\text{H}$ -thymidine uptake) of cultured lymphocytes from twenty-one diabetics who showed significant transformation to intact insulin

Study No.	Control cpm	Bovine A chain		Porcine A chain		Bovine B chain	
		cpm	T.I.	cpm	T.I.	cpm	T.I.
49	970	942	0.97	739	0.76	680	0.70
47	1,004	1,104	1.10	1,670	1.69	944	0.94
38	1,150	1,220	1.06	781	0.68	1,380	1.20
*58	902	939	1.04	747	0.83	1,136	1.26
41	1,623	1,445	0.89	-	-	2,173	1.34
45	1,684	1,161	0.69	-	-	2,339	1.39
36	1,092	963	0.88	-	-	1,527	1.40
40	870	938	1.08	523	0.60	1,313	1.51
*60	1,422	1,466	1.03	-	-	2,415	1.70
40	759	639	0.84	834	1.10	1,364	1.80
43	1,670	1,385	0.83	-	-	3,173	1.90
*57	518	682	1.10	-	-	1,171	1.90
42	925	1,833	1.98	906	0.98	1,880	2.03
*59	1,062	1,010	0.95	1,094	1.03	2,123	2.10
26	1,566	1,598	1.02	1,690	1.08	3,445	2.20
*56	1,241	1,450	1.17	-	-	6,452	5.20
39	950	1,020	1.07	-	-	5,605	5.90
37	860	1,039	1.20	-	-	6,192	7.20
48	1,287	1,364	1.06	1,094	0.85	11,866	8.70
25	705	750	1.05	-	-	6,201	8.80
46	964	972	1.01	630	0.66	9,350	9.70

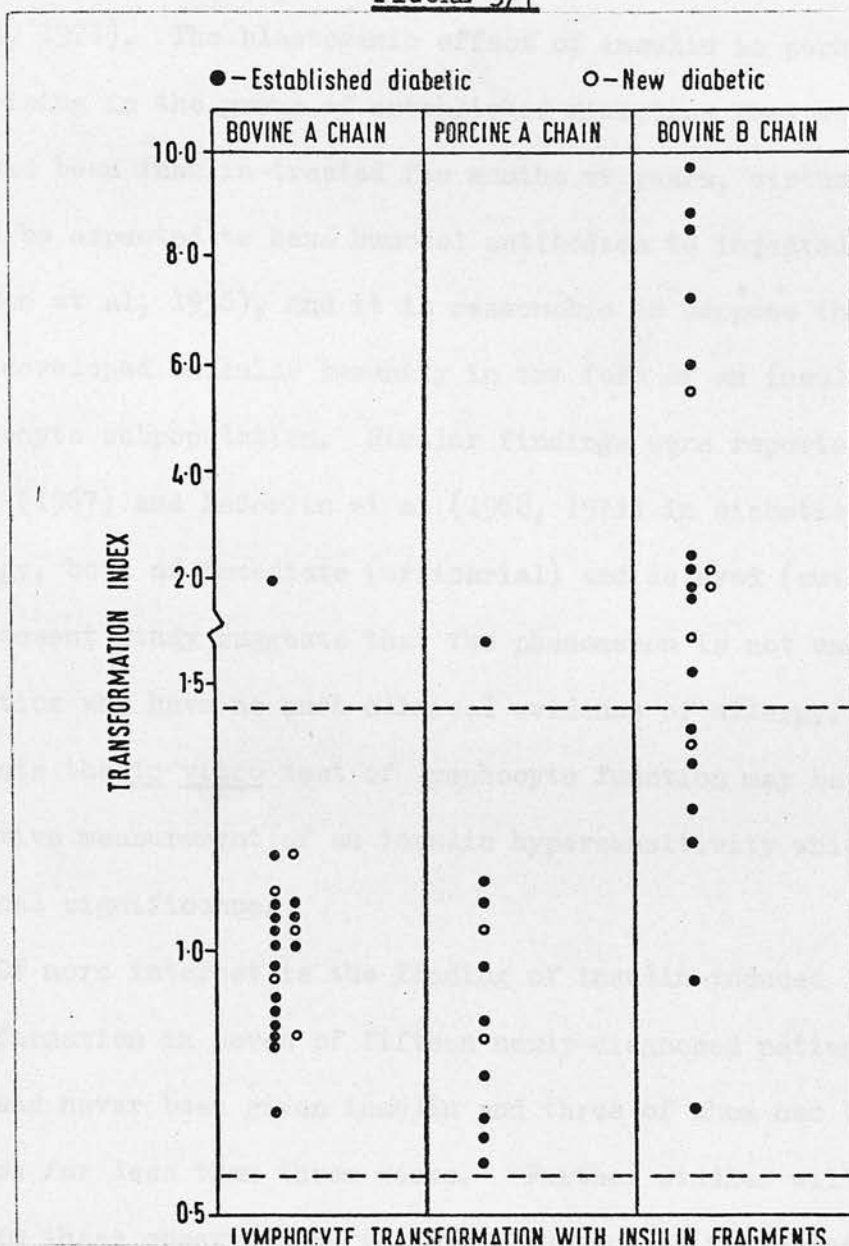
\* newly diagnosed patient.

'Study numbers' identify patients in Tables 3/7 and 3/8.

Results as in Table 3/6.

From MacGuish et al (1975)

FIGURE 3/7



Lymphocyte transformation induced by bovine and porcine A chain and bovine B chain in twenty-one diabetics who responded to intact insulin. Symbols as in Figure 4/5.

Closed circles (●) - established diabetics.

Open circles (○) - newly diagnosed diabetics.

Significant transformation to either A chain is shown by only one diabetic, as compared with fourteen (67%) who respond to B chain. The effects of B chain differ significantly ( $p < 0.01$ ) from those of the A chain of either insulin.

From MacGuish et al (1975)

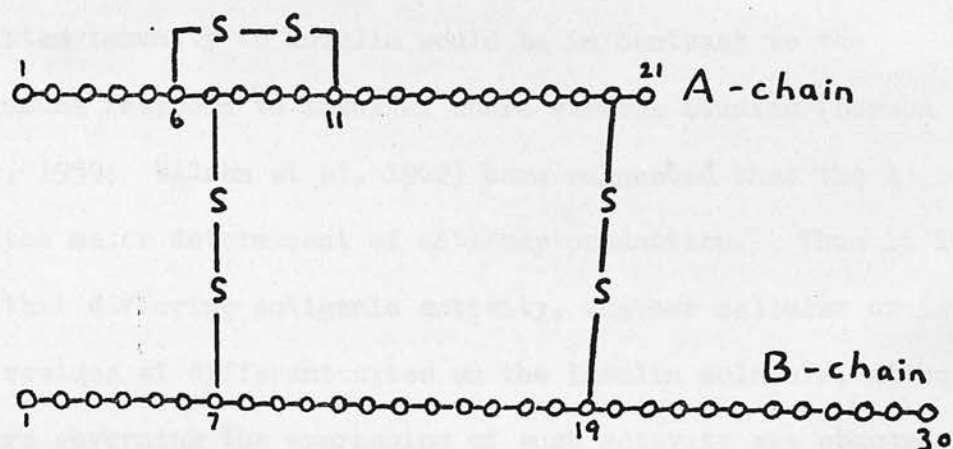


on lymphocytes from patients with Hashimoto thyroiditis (Ehrenfeld et al, 1971). The blastogenic effect of insulin is perhaps not surprising in the group of established diabetics that were tested: all had been insulin-treated for months or years, virtually all would be expected to have humoral antibodies to injected insulin (Berson et al, 1956), and it is reasonable to suppose that some have developed cellular immunity in the form of an insulin-sensitised lymphocyte subpopulation. Similar findings were reported by Halpern et al (1967) and Federlin et al (1968, 1971) in diabetics with insulin allergy, both of immediate (urticarial) and delayed (cutaneous) type; the present study suggests that the phenomenon is not uncommon in diabetics who have no such clinical evidence of allergy. In these patients the in vitro test of lymphocyte function may be the most sensitive measurement of an insulin hypersensitivity which is not of clinical significance.

Of more interest is the finding of insulin-induced lymphocyte transformation in seven of fifteen newly-diagnosed patients, four of whom had never been given insulin and three of whom had been insulin-treated for less than three weeks. Further studies will be needed to confirm these observations in larger numbers of untreated diabetics but the present results at least suggest that a proportion of such patients have an insulin-sensitised lymphocyte population before exogenous insulin has been given or before humoral insulin antibodies have been developed. Thus the existence of this cell population may reflect a state of cell-mediated autoimmunity, to insulin or insulin precursor, in early (untreated) diabetes; whether the phenomenon is analogous to the inhibition of leucocyte migration by pancreatic antigens/

antigens in similar patients, or whether these two in vitro tests are identifying different populations of sensitised lymphoid cells, is at present entirely speculative.

The virtually identical findings with bovine and porcine insulin prompted an examination of their component chains for antigenic effects, and two conclusions are possible from the results obtained. In the first place, the striking blastogenic effect of isolated B chain (which is metabolically inert) strongly implies that the blastogenesis induced by intact insulin is a true immunological effect, rather than some metabolic effect brought about by an hormonal action of insulin on the insulin receptors of lymphocytes. Secondly, there is a clearcut difference between the effects on the two major polypeptide chains of insulin. On the one hand, neither bovine nor porcine A chain induced significant lymphocyte transformation, and as the structure of human insulin A chain is identical to that of porcine insulin (Smith, 1966; figure 3/8) it might be presumed that human A chain would also be without blastogenic effect. On the other hand bovine insulin B chain, which is identical to that of porcine insulin and differs from the amino-acid sequence of human B chain only at position 30 (figure 3/8) had a marked effect in inducing blastogenesis of lymphocytes from two-thirds of those patients who responded to intact insulin. The results might thus suggest that B chain is the major antigenic site producing cellular hypersensitivity to insulin. Some support for this hypothesis is given by the in vivo animal experiments of Clark and Munoz (1970), who injected guinea pigs with bovine insulin, A and B chain in Freund's adjuvant: cutaneous hypersensitivity was readily elicited by/

FIGURE 3/8

	<u>A<sub>8</sub> - A<sub>9</sub> - A<sub>10</sub></u>	<u>B<sub>30</sub></u>
Beef Insulin	Ala - Ser - Val	Ala
Pig Insulin	Thr - Ser - Ileu	Ala
Human Insulin	Thr - Ser - Ileu	Thr

The primary structure of mammalian insulin. The major species difference between beef and porcine insulin resides in the A chain and in the amino-acid sequence within the intra-chain disulphide bridge. Human insulin is very similar to porcine insulin, having an identical A chain and differing only in the terminal amino-acid (30) of the B chain.

From the results of Smith (1966)

by intact insulin and by B chain, but A chain had no significant effect.

If the hypothesis is correct, the role of insulin B chain in cell-mediated immunity to insulin would be in contrast to the humoral immune response to insulin, where various studies (Berson and Yalow, 1959; Wilson et al, 1962) have suggested that the A chain is the major determinant of antibody production. Thus it is possible that differing antigenic activity, whether cellular or humoral, resides at different sites on the insulin molecule, although the factors governing the expression of such activity are obscure. However it must be remembered that an equally plausible explanation for the apparent lack of cellular antigenicity of A chain might lie in the physical changes undergone by this peptide during the splitting of insulin: isolated A chain is 'stretched' by comparison with its configuration in the intact insulin molecule, and the intra-chain disulphide bridge between six and eleven is broken with the formation of cysteic acid residues which could prevent antigen-antibody reaction at this site.

Further studies should attempt to define how commonly cell-mediated autoimmunity to insulin is found in untreated diabetics; to delineate more clearly the cellular antigenic portion of insulin; to indicate whether related proteins or precursors (e.g. proinsulin, C-peptide) have similar properties in early diabetes; and to correlate the results with antigen-induced migration inhibition studies in the same patients.

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Parts of this work have already been published: (MacCuish et al, 1974b, 1975)



## CHAPTER FOUR

### THE CORRELATION OF CELL-MEDIATED AND HUMORAL AUTOIMMUNITY IN DIABETES

#### 4/1 Introduction and purpose of study

The investigations described in this chapter were undertaken in a preliminary attempt to correlate the existence of humoral and cellular autoimmune phenomena in the same diabetic patients. For this purpose, diabetics with identifiable evidence of humoral antipancreatic autoimmunity (as judged by the possession of circulating cytoplasmic islet-cell antibodies) were tested to see whether they also had evidence of cellular antipancreatic immunity (as judged in vitro by their response to human pancreatic antigen in the leucocyte migration test). The results were compared with those given by a matched group of diabetics who did not possess islet-cell antibody, and with a control group of normal subjects.

#### 4/2 Selection of diabetics for study

A total of thirty-eight subjects were studied, subdivided into the following groups:

Insulin-dependent antibody-positive diabetics. This group consisted of ten patients with circulating pancreatic cytoplasmic islet-cell antibodies (chapter two). There were six women and four men, aged from twenty-one to fifty-two years (mean 35.6). All were insulin-taking patients, the duration of clinical diabetes ranging from four months to ten years and the age of onset from fifteen to forty-two years. Six had a clinical autoimmune disease in addition to diabetes; four had circulating thyrogastric antibodies but no clinical autoimmune disease. The relevant details of these patients are summarised in/

in table 4/1.

Insulin-dependent antibody-negative diabetics. This group comprised ten diabetics who did not possess circulating islet-cell antibodies. They were matched as closely as possible with the antibody-positive group with respect to age, sex and duration of insulin-dependent diabetes.

Normal controls. There were ten healthy nondiabetic volunteers (hospital and laboratory personnel) who were selected by sex and age to allow valid statistical comparison with the previous two groups. All were negative for islet-cell antibody.

Untreated juvenile-onset diabetics. The opportunity was also taken to study eight juvenile-onset diabetics who had never taken insulin or oral hypoglycaemic agents. Four were women and four men, aged from fourteen to thirty-six years (mean 22.3). All were newly-diagnosed and untreated at the time of study; three were negative for islet-cell antibody.

All the patients in the above groups were attending the Diabetic Department, Edinburgh Royal Infirmary, and (with the exception of the newly-diagnosed) all were well-controlled at the time of study.

#### Antigen used for study

The antigen used for these studies consisted of an islet-cell adenoma (insulinoma), weight 12.7g, which was removed from the pancreas of a twenty-five year old woman who presented with symptoms of spontaneous hypoglycaemia and who was found to have high fasting levels of plasma insulin. The tumour was made available by the courtesy of Mr. A.C.B. Dean (Consultant Surgeon, Royal Infirmary of Edinburgh), was cut into small pieces and snap-frozen immediately after removal, and/

TABLE 4/1

Ten insulin-dependent diabetics, with positive islet-cell antibodies,  
studied by the IMT using human insulinoma antigen

Age & Sex	Duration of Diabetes	Clinical Autoimmune Disease	Autoantibodies				
			Thg (titre)	TC	GPC	Adr (titre)	ANA
21/f	1.5 yrs	-	-	-	+	-	+
24/f	9.0 yrs	-	-	-	+	-	-
26/m	0.3 yrs	Addison's disease	-	-	-	32	-
26/m	2.5 yrs	-	25	+	-	-	-
35/f	3.5 yrs	Thyrotoxicosis	-	+	-	-	-
37/m	8.5 yrs	-	250	+	-	-	-
38/f	12 yrs	Thyrotoxicosis	-	-	-	-	-
48/f	6.5 yrs	Hashimoto Thyr.	2500	+	+	-	-
49/m	9.0 yrs	Pernicious anaem.	-	+	+	-	-
52/f	10 yrs	Hashimoto Thyr.	250	+	+	-	-

Thg = antibody to thyroglobulin

TC = antibody to thyroid cytoplasm

GPC = antibody to gastric parietal-cell cytoplasm

Adr = antibody to adrenal cortex

ANA = antibody to cell nuclei

+ = antibody present, titre not determined

- = antibody not present

and was studied extensively before being considered as a suitable antigen. Histological examination was undertaken by Dr. D. Thomson (Senior Lecturer in Pathology, University of Edinburgh): this confirmed the tissue to be an encapsulated, well-differentiated, benign islet-cell tumour, with staining reactions and morphology closely approximating to those of normal human islets when examined both by light and electron microscopy. Biochemical analysis of the tumour, and immunoassay of its hormone content, was kindly undertaken by Professor D.F. Steiner (Professor of Biochemistry, University of Chicago), to whom a 1.0g fraction of the specimen was sent: these studies demonstrated a tumour insulin content approximating to that of normal islet tissue; a proinsulin content (11%) within the high normal range; and a normal electrophoretic mobility of the insulin separated from the tumour by polyacrylamide gel electrophoresis. All these studies provided good evidence for regarding the structure and hormone content of this tumour as being virtually identical with that of normal islet tissue and the material was felt to be an ideal antigen for use in the leucocyte migration test, being an antigen of almost pure endocrine pancreas. Accordingly the whole tissue was homogenated, and after lyophilisation was stored in small batches at  $-40^{\circ}\text{C}$ ; each batch was used at a final protein concentration of 400  $\mu\text{g/ml}$  culture medium, this being the highest nontoxic concentration as determined by pilot experiments with normal controls.

#### Leucocyte migration test (LMT)

The LMT was performed exactly as described in the migration inhibition studies presented in chapter three of this thesis, i.e. by the method of Bendixen and Soborg (1969) with minor modifications.

The/



The effects of antigen on cell migration (the 'migration index') were again expressed as a percentage of migration without antigen using the formula:

$$\text{Migration index} = \frac{\text{Mean migration with antigen}}{\text{Mean migration without antigen}} \times 100\%$$

The normal range of migration indices using human islet-cell antigen was derived from the mean migration index  $\pm$  two standard deviations of the results given by the ten control subjects to this antigen at a concentration of 400  $\mu\text{g/ml}$  culture medium. The range in normals by this method was found to be 76.9% - 122.9% (mean 99.9  $\pm$  23): thus a figure of less than 76% was taken to indicate significant inhibition of leucocyte migration and above 123% significant stimulation.

All patients and controls were tested with the human islet-cell antigen. The results in the different subgroups were compared statistically by the Wilcoxon rank sum test (Wilcoxon, 1947).

#### 4/3 Results of study

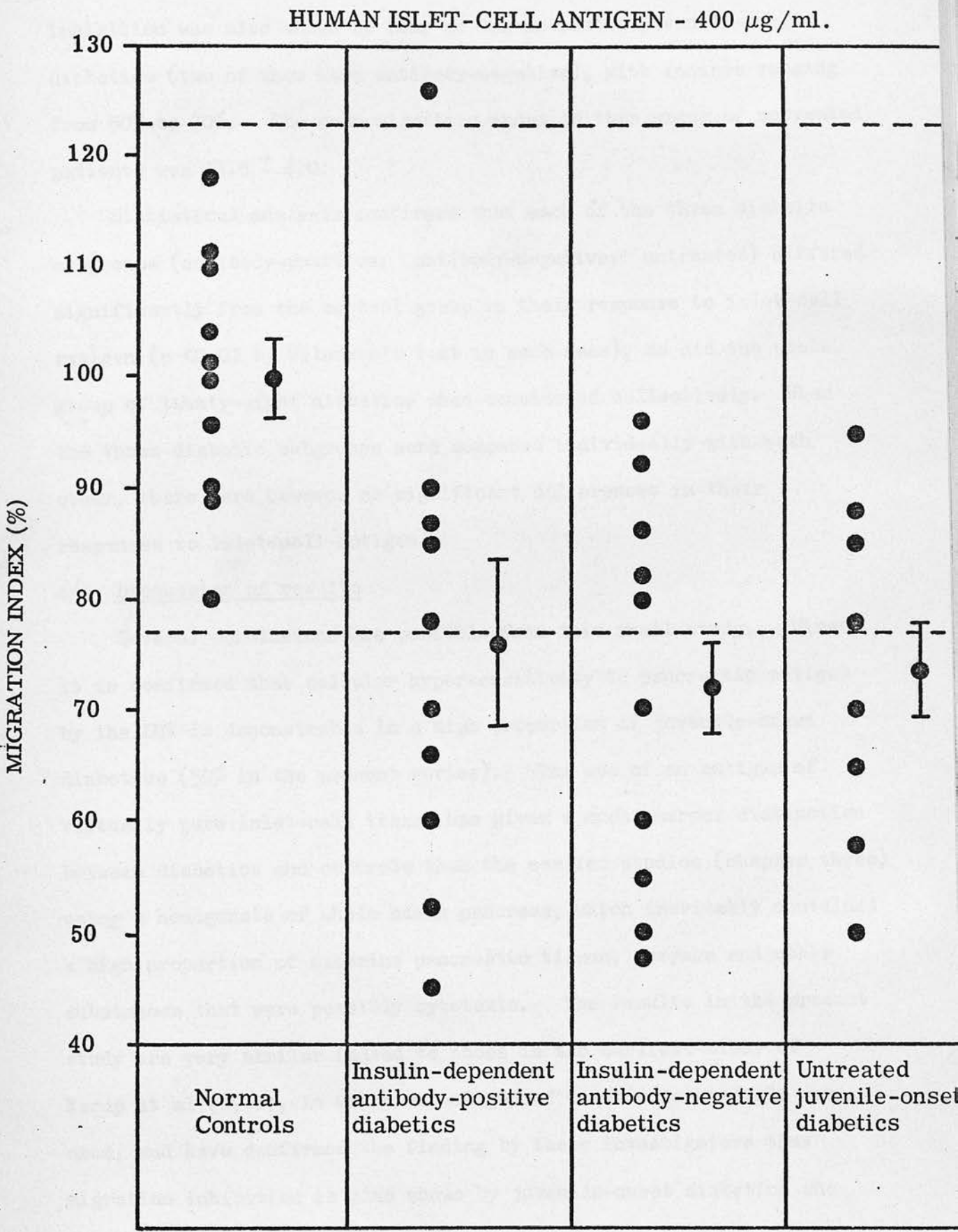
The results of leucocyte migration tests in diabetics and controls are presented in figure 4/1. None of the controls showed significant inhibition to human islet-cell antigen, the mean migration index in this group being 99.9  $\pm$  3.6 S.E.M. Five of the antibody-positive insulin-dependent diabetics showed significant inhibition, with indices ranging from 70% to 45%, and one showed significant stimulation of migration (index 126%) indicating weak hypersensitivity to islet-cell antigen. The mean migration index in these antibody-positive patients was 75.7  $\pm$  7.2. Very similar results were given by the matched group of antibody-negative insulin-dependent diabetics (mean index 71.9  $\pm$  4.1), five of whom showed significant inhibition to islet-cell/



FIGURE 4/1 (OPPOSITE):

Leucocyte migration tests with human islet-cell antigen (400 ug/ml) in normal controls, insulin-dependent diabetics with positive islet-cell antibody, insulin-dependent diabetics with negative islet-cell antibody, and untreated insulin-dependent diabetics. Significant inhibition (see text) is shown by fourteen (50%) of the diabetics and none of the controls. The three diabetic groups do not differ significantly.

FIGURE 4/1



From Irvine et al (1976b)



islet-cell antigen with indices ranging from 70% to 48%. Significant inhibition was also shown by four of the untreated juvenile-onset diabetics (two of whom were antibody-negative), with indices ranging from 50% to 70%. The mean migration index in this group of untreated patients was  $73.5 \pm 4.0$ .

Statistical analysis confirmed that each of the three diabetic subgroups (antibody-positive; antibody-negative; untreated) differed significantly from the control group in their response to islet-cell antigen ( $p < 0.01$  by Wilcoxon's test in each case), as did the whole group of twenty-eight diabetics when considered collectively. When the three diabetic subgroups were compared individually with each other, there were however no significant differences in their responses to islet-cell antigen.

#### 4/4 Discussion of results

Several conclusions are possible from this small study. First, it is confirmed that cellular hypersensitivity to pancreatic antigen by the LMT is demonstrable in a high proportion of juvenile-onset diabetics (50% in the present series). The use of an antigen of virtually pure islet-cell tissue has given a much sharper distinction between diabetics and controls than the earlier studies (chapter three) using a homogenate of whole human pancreas, which inevitably contained a high proportion of exocrine pancreatic tissue, enzymes and other substances that were possibly cytotoxic. The results in the present study are very similar indeed to those in the earliest study of Nerup et al (1971), in which an antigen of porcine islet-cells was used, and have confirmed the finding by these investigators that migration inhibition is also shown by juvenile-onset diabetics who have/

have never received insulin or indeed any other hypoglycaemic drug therapy. The findings in these patients give credence to the belief that cellular hypersensitivity to pancreatic antigens in young diabetics is a true autoimmune phenomenon, i.e. independent of previous anti-diabetic treatment; they are quite different from the responses of non-insulin-dependent diabetics, very few of whom show cellular hypersensitivity to pancreatic antigens (Nerup et al, 1973, 1974; chapter three).

So far as the author is aware, the present investigation was the first study in which the presence of humoral autoimmune phenomena (possession of pancreatic islet-cell antibody) and cellular autoimmunity (hypersensitivity to islet-cell antigen) were directly compared in the same diabetic patients. The results (figure 4/1) indicate that cellular hypersensitivity is not consistently present in antibody-positive diabetics, being found in only half such patients studied, and that cellular hypersensitivity may equally be found in a similar proportion of diabetics who do not possess this humoral islet-cell antibody. These findings would suggest that the correlation between humoral and cellular autoimmunity in young diabetics is relatively poor and two subsequent studies have supported this conclusion. Christy et al (1976) found no correlation between cytoplasmic ICA and cell-mediated autoimmunity in a group of thirty-eight 'juvenile diabetics' (presumably insulin-dependent) with a mean disease duration of 3.2 years. Richens et al (1979) reported a similar finding in a group of forty-nine young diabetics, again with disease duration of 3.2 years, where a simultaneous search was made for cytoplasmic ICA and evidence of cell-mediated autoimmunity by the IMT/

LMT. It is clear that both Christy et al and Richens et al employed relatively crude antigens in the LMT - homogenates of whole pancreas or liver mitochondrial preparations - and this probably explains the low frequency of migration inhibition (about 25% of each group of diabetic patients) observed by these workers. Nevertheless, all three studies might suggest that cytoplasmic ICA is not an invariable 'marker' of other autoimmune phenomena in diabetes.

However it must be remembered that cytoplasmic ICA are only detectable (by immunofluorescence) in comparatively low titres and that the frequency of positive results varies not only between different laboratories but also with the source and fixation of the tissue substrate used: for example, it is possible that paraffin-embedded sections of human group O pancreas as described by Dobersen et al (1979) would have yielded a higher number of antibody-positive subjects. These kind of difficulties are only likely to be resolved when standardised pancreatic endocrine cell lines are generally available for use as a substrate in serological testing for antibodies, and the other measures suggested in chapter two are taken to standardise the equipment used for antibody detection. Alternatively, a different objective method might be developed for antibody assay, such as radio-immunoassay, or measurement of another of the pancreatic islet-cell antibodies (perhaps islet-cell surface antibody) might yield a better correlation. Precisely the same reservations apply to antigens used to demonstrate cell-mediated antipancreatic autoimmunity by the LMT: the study described here used the purest possible concentration of beta cells obtainable from human source but even this was tumour material, and the antigens used in the other two studies were relatively crude/

crude homogenates. Again this objection could be answered when pure preparations of pancreatic cell lines become available.

Another consideration is the possibility that the one hundred and fifteen insulin-dependent diabetics described here (twenty-eight tested by the author, thirty-eight by Christy et al, forty-nine by Richens et al) were genetically and aetiologically heterogeneous for Type 1 diabetes. HLA typing was not performed in the patients tested by the author but was undertaken by both other groups, and Christy et al were able to demonstrate a positive association between HLA-B8 and antipancreatic autoimmunity, both humoral and cellular. Richens et al tested for A and B locus antigens but found only seventeen antibody-positive patients and were unable to draw statistical conclusions from such a small number. The association between HLA-B8 (and probably DR3) with the persistence of islet-cell antibody and with coexistent overt autoimmune disease has already been reviewed (chapter two; see also chapter seven) and it is possible that the subgroup of true 'autoimmune Type 1 diabetes' will be identified by performing HLA typing in addition to testing for antipancreatic humoral and cell-mediated immunity in these patients.

In summary, it appears that the question of a correlation between in vitro tests for antipancreatic humoral and cell-mediated autoimmune phenomena could be accurately examined by the following future studies:

1. HLA typing of appropriately large number of insulin-dependent (Type 1) diabetics at the onset of clinical disease, and separation of these patients into those who are HLA-DR3 positive and HLA-DR4 positive.
2. Examining both subgroups for the presence of cytoplasmic ICA, ICSA or other pancreatic antibodies with standardised assays using antigens/



antigens of known composition, perhaps derived from specific endocrine pancreatic cell lines.

3. Testing both groups for evidence of antipancreatic cell-mediated immunity, again using antigens of known composition (perhaps the same antigens used as substrate for the humoral antibody detection).

4. Comparing the results of these in vitro tests for autoimmunity with in vivo evidence of antipancreatic autoimmunity (for example, the induction of delayed-type cutaneous hypersensitivity to injections of antigens used in the in vitro studies).

It is possible that a study of this nature would not only extend our knowledge of autoimmune phenomena in diabetes mellitus but might also define a subgroup of Type 1 diabetics in whom the condition could be accepted as a true autoimmune disease.

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Part of this work has already been published (Irvine, MacCuish, Campbell and Duncan, 1976b).

## CHAPTER FIVE

## STUDIES OF GENERAL CELL-MEDIATED IMMUNE FUNCTION IN DIABETES

5/1 Introduction and purpose of studies

The mitogen phytohaemagglutinin (PHA) induces transformation of cultured small lymphocytes to blast cells (Nowell, 1960). Under appropriate conditions, 60-70% of lymphocytes are transformed in vitro by PHA and the majority of these cells will be T lymphocytes (Roitt et al, 1969) although some B lymphocytes are also stimulated to transform (Phillips and Roitt, 1974). PHA testing is therefore extensively employed as a general assessment of cell-mediated immune function in a variety of clinical states and may be regarded as a convenient means of judging the functional level of the recirculating pool of lymphocytes, especially helper T cells (Roitt, 1980).

Conflicting results have been reported by investigators who have undertaken PHA testing in diabetes. Thus Brody and Merlie (1970) reported profound depression of PHA response in six diabetics, five of whom were insulin-dependent, and claimed that the direct oxidative pathway of glucose metabolism was also abnormal; these results therefore suggested immunological abnormality in diabetes. By contrast Ragab et al (1972) tested the PHA response of lymphocytes from twenty-three diabetics, seventeen of whom were insulin-dependent, and found no difference from the response of cells from twenty-four healthy nondiabetic controls in the same assay system. Ragab et al further measured the lymphocyte transformation response to a commonly-encountered antigen (*Candida albicans*) in their diabetic and control groups, and again found the response of each group to be identical. Thus/

Thus the results of Ragab et al suggested that general cell-mediated immune function was normal in diabetes mellitus.

On reviewing the work of Brody and Merlie and Ragab et al, it seemed possible that the differing findings of these two groups could perhaps be reconciled by relating PHA response to the degree of metabolic control in insulin-dependent diabetes. With this possibility in mind, the following studies of general cell-mediated immune function in diabetics were therefore undertaken:

1. The transformation response to PHA was measured using lymphocytes from both well-controlled and poorly-controlled insulin-dependent diabetics, the results being compared with those obtained from matched nondiabetic controls.
2. The circulating subpopulations of T lymphocytes (which constitute the main bulk of PHA-responsive cells) and B lymphocytes were also measured in peripheral blood from the same diabetics and controls.

When these initial investigations had indicated that the magnitude of PHA response in diabetic lymphocyte cultures was indeed related to the adequacy of metabolic control, further studies were undertaken in an attempt to define the factor(s) responsible for lymphocyte malfunction in poorly-controlled diabetes mellitus.

#### 5/2 Selection of well-controlled and poorly-controlled diabetics for study

PHA responses were studied in forty well-controlled diabetic and forty normal subjects who were carefully matched for age and sex. The diabetic subjects (twenty-two women, eighteen men; mean age 42.4 years) were insulin-dependent, attending the outpatient clinic of the Diabetic Department, Edinburgh Royal Infirmary, and were free from infection on the day of study. The control subjects were healthy volunteers/

volunteers, mainly laboratory personnel or hospital outpatients who were not known to have endocrine disease or immunologic abnormality.

PHA responses were also studied in a group of fourteen poorly-controlled insulin-dependent diabetics. These patients had been brought to the outpatient clinic for routine or emergency review and their disorder was judged to be poorly controlled by the following criteria: midmorning blood glucose exceeding 20 mmol/l (350 mg/dl); recent increase in insulin requirements; the presence of heavy glycosuria and/or ketonuria. Some were subsequently admitted to hospital for correction of their metabolic abnormalities but none had received antibiotics or drugs other than insulin at the time of study. Their clinical details are presented in table 5/1.

T and B cell subpopulations were measured in fifteen of each of the well-controlled diabetic and normal subjects, and in ten of the poorly-controlled diabetics.

### 5/3 Methods used for collection, culture and identification of lymphocytes

#### Collection of blood samples and separation of lymphocytes

Venous blood was withdrawn at midmorning and anticoagulated with preservative-free heparin (Weddell Pharmaceuticals or Evans Medical). A portion of each sample was used for measurement of total and differential white blood cell counts. Lymphocytes were separated from the remainder by density centrifugation on a Ficoll-Trisil gradient (Calder et al, 1973; Appendix I/lb) and washed three times in Eagle's Basal Medium (EBM, Appendix I/lc). The cells were then resuspended in EBM with 10% foetal calf serum (Wellcome), counted, and/



TABLE 5/1

Clinical details of fourteen poorly-controlled diabetic patients  
in whom lymphocyte transformation and lymphocyte subpopulation  
studies were undertaken.

Age/ Sex	Duration of diabetes (yrs)	Blood glucose (mg per cent)	Cause of poor control
24/f	10.0	358	Depressive illness
37/f	0.3	355	Recent diagnosis, establishing control
48/f	5.2	450	Urinary tract infection
60/f	16.0	355	Emotional stress
61/f	11.0	352	Upper respiratory tract infection
66/f	5.0	440	Foot infection
68/f	0.05	385	New diagnosis, establishing control
69/f	7.1	650	Congestive cardiac failure
17/f	6.9	510	Ketoacidosis, cause unknown
19/m	10.0	365	Skin sepsis, chest infection
28/m	13.0	408	Alcoholic, drinking bout
45/m	6.0	400	Acute bronchitis
46/m	9.0	360	Dental infection
60/m	3.7	800	Bronchopneumonia

From MacCuish et al (1974c)

and the concentration adjusted to  $1 \times 10^6$  cells per ml. All cell suspensions contained more than 95% lymphocytes with a viability greater than 98% on trypan blue exclusion. Lymphocytes from the same sample were used for the PHA test and for T and B cell estimations.

#### Lymphocyte culture with PHA

The lymphocyte transformation response to PHA was measured by a microculture technique using radioisotope labelling of stimulated cells. The technique was developed in the MRC Clinical Immunology Laboratories, Forrest Road, Edinburgh, under the direction of Dr. W.J. Penhale (Senior MRC Research Worker); prior to its use the method had been extensively investigated to provide a rapid, reproducible and inexpensive technique for PHA-induced lymphocyte transformation, using only small quantities of lymphocytes and ensuring minimal radiation damage to the cells. The technique (Penhale et al, 1974) is described in detail in Appendix II/4 of this thesis and is summarised as follows: Stock PHA (PHA-P, Difco) was diluted in EBM with 10% foetal calf serum to give three solutions containing respectively 0.32, 0.63 and 1.25  $\mu$ l PHA/ml culture. Aliquots of 20  $\mu$ l of each solution were pipetted into the wells of Cooke Microtiter trays (Flow Laboratories) and  $2 \times 10^5$  cells added to each well. All determinations were performed in triplicate and included control cultures without PHA. The microtrays were gassed with an air-5%  $\text{CO}_2$  mixture and incubated at  $37^\circ\text{C}$  in sealed, humidified containers.

$^3\text{H}$ -thymidine of specific activity 5 Ci/mmol (Radiochemical Centre, Amersham) was diluted with sterile saline to give a working concentration/

concentration of a 2  $\mu\text{Ci}/100 \text{ ul}$ . Twenty microlitres of this solution (i.e. 0.4  $\mu\text{Ci}$   $^3\text{H}$ -thymidine) was added to each culture after forty-four hours' incubation. The cultures were regassed and incubated for a further four hours. The contents of the wells were then pipetted onto glass fibre filter papers (Whatman GF/C) which were air-dried, washed successively with cold 5% trichloroacetic acid, phosphate buffered saline (Appendix I/1) and absolute methanol, and finally placed in Packard glass counting vials. Five ml Scintillation fluid (NE233, Nuclear Enterprises Ltd.) was added to each vial and the samples counted for sixty seconds in an automatic beta counter (Packard Tricarb 2425), the results being expressed as counts per minute (cpm).

#### Identification of T and B lymphocyte subpopulations

These investigations were kindly undertaken by Dr. S.J. Urbaniak during his tenure of an MRC Junior Research Fellowship in the Clinical Immunology Laboratories under Dr. Irvine's direction. T lymphocytes were identified by their ability to form rosettes with sheep erythrocytes (E-rosettes). The technique used (Urbaniak et al, 1973) was derived from that of Jondal et al (1972) and incorporated the modifications of Stjernsward et al (1972). Results are expressed both as absolute numbers and percentages of total lymphocyte counts.

B lymphocytes were identified by two techniques: a rosette technique (Stjernsward et al, 1972), using sheep red cells coated with antibody and complement (EAC-rosettes) and indirect immunofluorescence (Jondal et al, 1972), whereby the cells are distinguished by surface immunoglobulin marker.

#### 5/4 Results of lymphocyte transformation with PHA

The dose-response curves of lymphocytes from well-controlled diabetics/

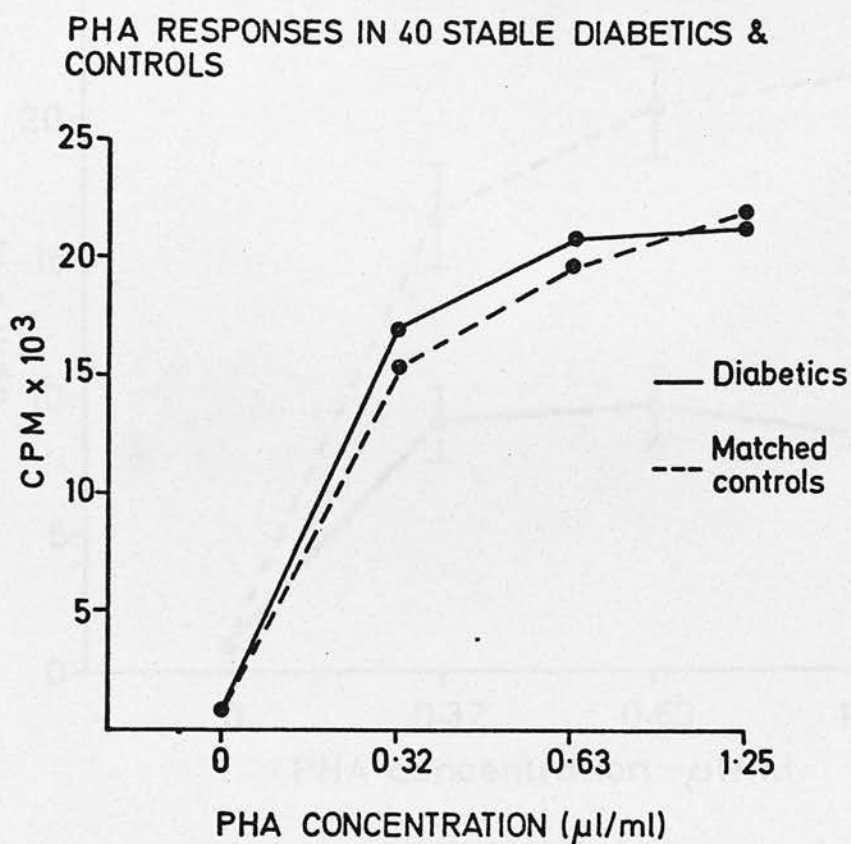
diabetics and normal subjects are shown in figure 5/1. The mean transformation responses in the diabetics to the three doses of PHA employed were  $17.0 \pm 4.3$ ,  $20.8 \pm 4.2$  and  $21.4 \pm 4.3$  respectively (expressed as  $\text{cpm} \times 10^3 \pm \text{S.E.M.}$ ). The corresponding values in the controls were  $15.3 \pm 4.3$ ,  $19.7 \pm 3.9$  and  $21.8 \pm 3.9$ . Both curves are virtually identical and the lymphocyte transformation response to PHA is not therefore abnormal in well-controlled diabetics.

The dose-response curve of lymphocytes from the fourteen poorly-controlled diabetics is compared in figure 5/2 with the curve obtained from fourteen of the well-controlled diabetics, matched as closely as possible for age, sex and duration of diabetes. The mean transformation responses in poorly-controlled diabetics to the three doses of PHA were  $9.0 \pm 1.33$ ,  $9.5 \pm 1.4$  and  $8.2 \pm 0.6$  respectively. The corresponding values in well-controlled diabetics were  $16.5 \pm 2.0$ ,  $20.2 \pm 1.9$  and  $21.3 \pm 2.0$ . These results show a statistically significant reduction of PHA response in poorly-controlled insulin-dependent diabetics at all three concentrations of PHA ( $p < 0.001$  by the Student t test).

#### 5/5 Results of lymphocyte subpopulation studies

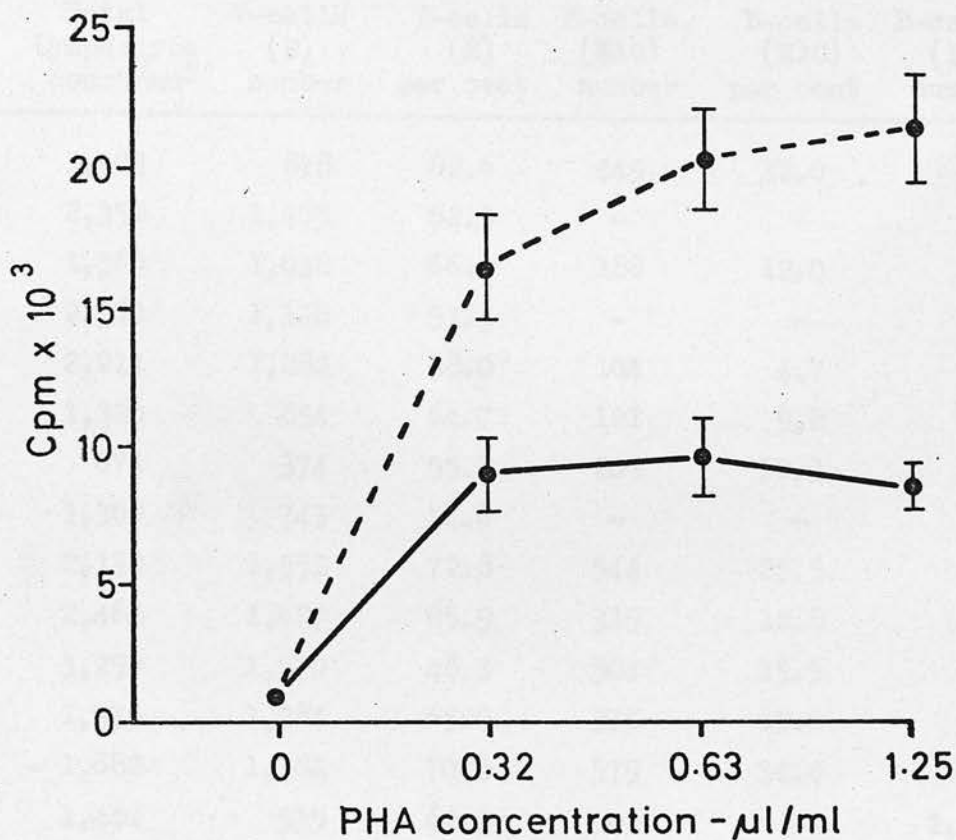
Tables 5/2, 5/3 and 5/4 list the total lymphocyte counts and the numbers of circulating T and B cells, expressed both as absolutes and percentages of total lymphocyte counts, in samples taken respectively from fifteen well-controlled diabetics, fifteen normal subjects and ten poorly-controlled diabetics. The results show no significance in the mean number and percentage of T cells (assessed by E rosettes) or B cells (assessed by EAC rosettes or immunofluorescence techniques). Circulating lymphocyte subpopulations are/



FIGURE 5/1

Mean lymphocyte transformation responses in forty well-controlled insulin-dependent diabetics (—) and forty age- and sex-matched normal subjects (---) at three concentrations of PHA. The two dose-response curves do not differ significantly. S.E.M.'s are omitted for clarity but are given in the text.

From MacGuish et al (1974c)

FIGURE 5/2

Lymphocyte transformation responses (mean  $\pm$  S.E.M.) in fourteen poorly-controlled insulin-dependent diabetics (—) and fourteen well-controlled insulin-dependent diabetics (---) who were matched for age, sex and duration of diabetes. The mean response in the poorly-controlled diabetics is significantly lower ( $p < 0.001$ ) at all concentrations of PHA.

From MacGuish et al (1974c)

TABLE 5/2

Total lymphocyte counts and subpopulations of T and B lymphocytes  
in peripheral blood from fifteen well-controlled insulin-dependent  
diabetic patients

Age/ Sex	Total Lymphocyte count/mm <sup>3</sup>	T-cells (E) number	T-cells (E) per cent	B-cells (EAC) number	B-cells (EAC) per cent	B-cells (1F) number	B-cells (1F) per cent
22/f	1,403	878	62.6	449	32.0	-	-
28/f	2,350	1,465	62.3	-	-	357	15.2
35/f	1,566	1,038	66.3	188	12.0	227	14.5
55/f	2,560	1,318	51.5	-	-	294	11.5
56/f	2,211	1,282	58.0	104	4.7	736	33.3
59/f	1,320	854	64.7	121	9.2	300	22.7
62/f	671	374	55.8	203	30.3	221	33.0
62/f	1,360	743	54.6	-	-	299	22.0
68/f	2,132	1,552	72.8	544	25.5	452	21.2
17/m	2,464	1,627	65.9	315	12.8	490	19.9
19/m	3,250	1,570	48.3	504	15.5	523	16.1
36/m	1,975	1,284	65.0	375	19.0	257	13.0
42/m	1,682	1,182	70.3	579	34.4	-	-
63/m	1,440	935	64.9	-	-	2,321	16.1
Mean	1,879	1,153	62.0	338	19.5	375	20.4
<sup>±</sup> S.E.M.	165	91	1.79	56	3.29	42	1.98

(E) = T lymphocytes identified by sheep erythrocyte rosettes

(EAC) = B lymphocytes identified by erythrocyte-antibody-complement rosettes

(1F) = B lymphocytes identified by indirect immunofluorescence

From MacCuish et al (1974c)

TABLE 5/3

Total lymphocyte counts and subpopulations of T and B lymphocytes  
in peripheral blood from fifteen normal subjects

Age/ Sex	Total Lymphocytes count/mm <sup>3</sup>	T-cells (E) number	T-cells (E) per cent	B-cells (EAC) number	B-cells (EAC) per cent	B-cells (IF) number	B-cells (IF) per cent
27/f	1,333	857	64.3	216	16.2	-	-
27/f	710	474	66.8	192	27.0	90	12.7
38/f	1,976	1,320	66.8	174	8.8	326	16.5
47/f	1,606	896	55.8	527	32.8	390	24.3
55/f	1,817	950	52.3	136	7.5	-	-
57/f	770	506	65.7	218	28.3	123	16.0
57/f	2,667	1,560	58.5	494	18.5	416	15.6
64/f	2,184	1,223	56.0	109	5.0	618	28.3
69/f	2,054	1,516	73.8	496	24.1	251	12.2
18/m	3,000	2,223	74.1	1,125	37.5	690	23.0
25/m	700	513	73.3	86	12.3	123	17.5
35/m	2,379	1,421	59.3	43	1.8	816	34.3
42/m	1,974	1,137	58.4	516	26.5	405	20.8
66/m	1,672	1,167	69.8	274	16.4	-	-
Mean	1,855	1,194	64.4	323	19.4	415	20.5
<sup>±</sup> S.E.M.	194	112	1.64	73	4.02	60	2.56

Abbreviations as for Table 5/2.

From MacCuish et al (1974c)



TABLE 5/4

Total lymphocyte counts and subpopulations of T and B lymphocytes  
in peripheral blood from ten poorly-controlled insulin-dependent  
diabetic patients

Age/ Sex	Total Lymphocyte count/mm <sup>3</sup>	T-cells (E) number	T-cells (E) per cent	B-cells (IF) number	B-cells (IF) per cent
24/f	1,035	735	71.0	207	20.0
37/f	2,700	2,173	80.5	259	9.6
60/f	1,936	1,073	55.4	407	21.0
61/f	2,430	1,638	67.4	676	27.8
66/f	2,436	1,571	64.5	470	19.3
68/f	1,850	1,166	63.0	487	26.3
19/m	600	303	50.5	102	17.0
28/m	1,472	883	60.0	202	13.7
45/m	1,971	1,242	63.0	242	12.3
46/m	1,950	1,225	62.8	558	28.6
Mean	1,838	1,201	63.8	361	19.5
<sup>±</sup> S.E.M.	145	109	1.59	38	3.25

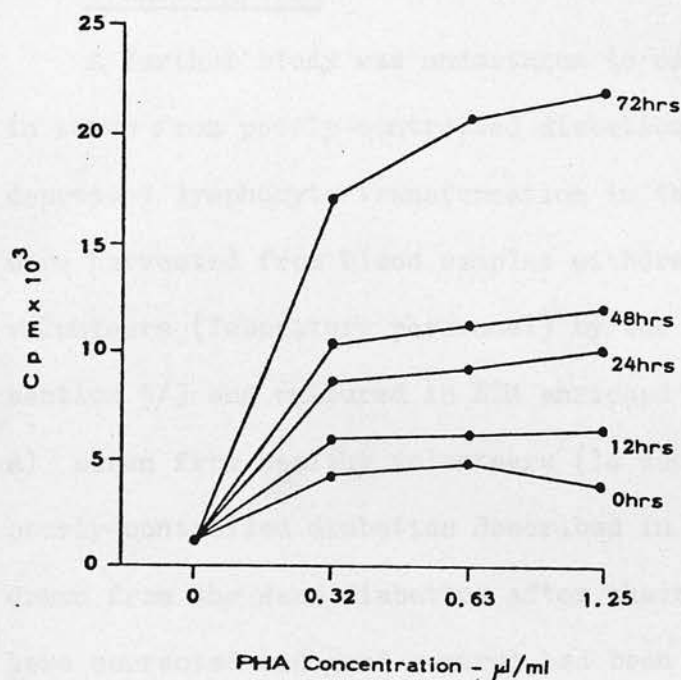
Abbreviations as for Table 5/2.

From MacGuish et al (1974c)

are therefore normal in diabetics when obtained at times of 'good' and 'poor' metabolic control.

#### 5/6 Serial measurements of lymphocyte transformation during recovery from diabetic ketoacidosis

From the results of the above studies, it appeared likely that some (transient) metabolic factor was causing depression of lymphocyte transformation in diabetics at times of poor diabetic control. Thus the opportunity was taken to make serial measurements of PHA-induced lymphocyte transformation in a diabetic during recovery from ketoacidosis. The patient studied was a man aged 36 years, with a history of insulin-dependent diabetes for ten years, who was admitted to Edinburgh Royal Infirmary in severe diabetic ketoacidosis. The cause of his metabolic decompensation was not established but no evidence of underlying infection was detected by bacteriological examination of the appropriate body fluids. Following admission he was treated intensively with intravenous fluids, electrolytes and insulin and made an uneventful recovery. Blood for study was withdrawn at the time of hospital admission (before treatment commenced) and at 12, 24, 48 and 72 hours thereafter, i.e. during the period of intensive medical therapy. Lymphocytes from the serial blood samples were harvested and cultured in the presence of PHA as described in section 5/3. The results are presented in figure 5/3, together with biochemical data (serial blood glucose and plasma bicarbonate concentrations) obtained at the same times. It will be observed that lymphocyte transformation with PHA was grossly depressed in severe diabetic metabolic decompensation but slowly returned to normal as biochemical improvement occurred. However the/

FIGURE 5/3

BIOCHEMISTRY		
Time	BG	HCO <sub>3</sub> <sup>-</sup>
0hrs	650	<5
12hrs	270	14
24hrs	124	21
48hrs	58	24.5
72hrs	110	23.5

BG - blood glucose (mg%)

HCO<sub>3</sub><sup>-</sup> - plasma bicarbonate (mEq/l)

Lymphocyte transformation responses to three concentrations of PHA in an insulin-dependent diabetic during recovery from ketoacidosis. Transformation response is grossly depressed in severe metabolic decompensation but returns to normal within 72 hours following correction of the ketoacidosis.

From MacGuish and Irvine (1975)

the lymphocyte transformation response remained markedly depressed at 48 hours, by which time the patient's blood glucose and plasma bicarbonate concentrations were both normal. After a further 24 hours, i.e. 72 hours after admission and treatment, the transformation responses to PHA had returned to normal.

#### 5/7 Effects of diabetic serum on mitogen-induced lymphocyte transformation

A further study was undertaken to establish that some factor in serum from poorly-controlled diabetics was responsible for the depressed lymphocyte transformation in these patients. Lymphocytes were harvested from blood samples withdrawn from a panel of healthy volunteers (laboratory personnel) by the techniques described in section 5/3 and cultured in EEM enriched with the following serum:

a) serum from healthy volunteers (14 samples); b) serum from the poorly-controlled diabetics described in table 5/1; c) serum withdrawn from the same diabetics after their metabolic abnormalities were corrected and good control had been established (11 samples). In all cases the serum was added to EEM in a concentration of 10% and PHA was also added to give a concentration of 0.63  $\mu$ l PHA/ml. All determinations were performed in triplicate. Control cultures from all three groups were simultaneously cultured in EEM with 10% foetal calf serum, again in the presence of PHA at a concentration of 0.63  $\mu$ l/ml. All results of culture were expressed as counts per minute (cpm); the difference between the transformation of lymphocytes cultured in human test serum and lymphocytes cultured in foetal calf serum was expressed as the 'Transformation Index' by the following formula:

$$\text{Transformation Index} = \frac{\text{cpm of cultures in test serum}}{\text{cpm of cultures in foetal calf serum}}$$

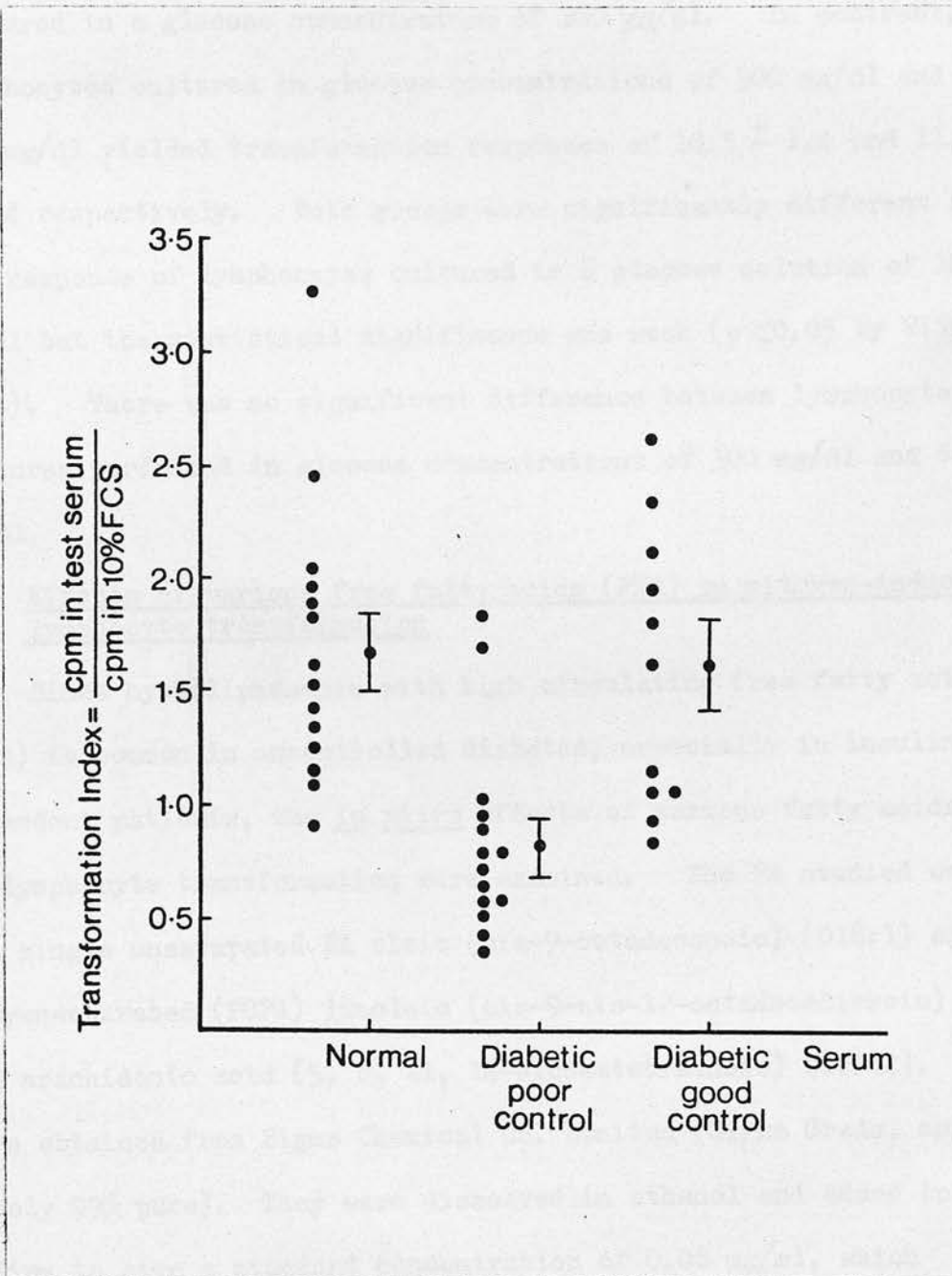
The/



The results of this experiment are shown in figure 5/4. The mean transformation index ( $\pm$  S.E.M.) was  $1.67 \pm 0.2$  for normal lymphocytes cultured in normal serum; using diabetic serum, the mean transformation index for normal lymphocytes was  $0.81 \pm 0.1$  when cultured with serum from poorly-controlled diabetics and  $1.60 \pm 0.23$  when cultured with serum taken from the same patients after good diabetic control had been established. Statistical analysis by the Wilcoxon rank test confirmed that there was no significant difference between the transformation of lymphocytes cultured in normal serum or in serum from well-controlled diabetics. However the transformation of lymphocytes cultured in serum from poorly-controlled diabetics was significantly lower ( $p < 0.01$ ) than both the other groups. These results indicate clearly that some factor(s) in the serum of poorly-controlled diabetics can inhibit the PHA-induced transformation of normal lymphocytes.

#### 5/8 Effects of varying glucose concentration on mitogen-induced lymphocyte transformation

This experiment was designed to examine the possibility that hyperglycaemia per se was responsible for reduced lymphocyte transformation in poorly-controlled diabetics. Lymphocytes were prepared from a panel of eight healthy volunteers and cultured as before in EEM with 10% foetal calf serum and PHA at a concentration of  $0.63 \mu\text{l/ml}$ . However the final glucose concentration of the culture medium was adjusted to give three solutions containing respectively glucose at 100, 300 and 600 mg/dl culture medium. (Normal EEM contains glucose at a concentration of 100 mg/dl). All cultures were performed in triplicate and included control cultures without PHA. The results were/

FIGURE 5/4

Effect of serum from fourteen normal subjects and fourteen diabetics on the PHA-induced transformation of normal lymphocytes. Serum from poorly-controlled diabetics causes significant ( $p < 0.01$ ) depression of lymphocyte transformation; repeat serum samples, obtained from the same patients when their diabetes was well controlled, have no such inhibitory effects of lymphocyte transformation.

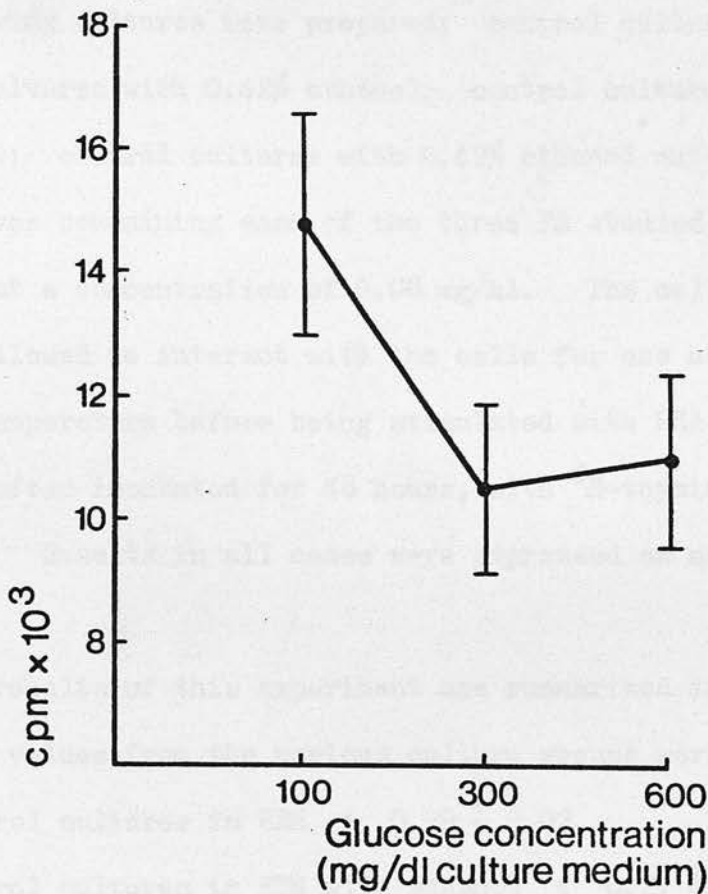
From MacGuish (1976)

were expressed as  $\text{cpm} \times 10^3$  and are shown in figure 5/5. The mean transformation response  $\pm$  S.E.M. was  $14.8 \pm 1.8$  for lymphocytes cultured in a glucose concentration of 100 mg/dl. In contrast, lymphocytes cultured in glucose concentrations of 300 mg/dl and 600 mg/dl yielded transformation responses of  $10.5 \pm 1.4$  and  $11.0 \pm 1.4$  respectively. Both groups were significantly different from the response of lymphocytes cultured in a glucose solution of 100 mg/dl but the statistical significance was weak ( $p < 0.05$  by Wilcoxon's test)!. There was no significant difference between lymphocyte cultures performed in glucose concentrations of 300 mg/dl and 600 mg/dl.

#### 5/9 Effects of various free fatty acids (FFA) on mitogen-induced lymphocyte transformation

Since hyperlipidaemia with high circulating free fatty acids (FFA) is common in uncontrolled diabetes, especially in insulin-dependent patients, the in vitro effects of various fatty acids (FA) on lymphocyte transformation were examined. The FA studied were the single unsaturated FA oleic (cis-9-octadecanoic) (C18:1) and the polyunsaturated (PUFA) linoleic (cis-9-cis-12-octadecadienoic) (C18:2) and arachidonic acid (5, 8, 11, 14-eicosatetraenoic) (C20:4). All were obtained from Sigma Chemical Co. Limited (Sigma Grade, approximately 99% pure). They were dissolved in ethanol and added to culture medium to give a standard concentration of 0.08 mg/ml, which corresponds to the normal level of C18:1 in human serum (Schrade et al, 1960). The ethanol concentration did not exceed 0.42% in the test culture medium and was also added at the same concentration to the FA-free controls.

Lymphocytes/

FIGURE 5/5

Effect of increasing glucose concentration on the transformation response to PHA (0.63  $\mu$ l/ml) of cultured lymphocytes from eight normal subjects. Glucose concentrations of 300 and 600 mg/dl have a small but significant ( $p < 0.05$ ) effect in depressing lymphocyte transformation. Mean PHA response  $\pm$  S.E.M. is shown for each glucose concentration.

From MacCuish (1976)



Lymphocytes for culture were obtained from twelve healthy volunteers (laboratory personnel) and prepared as described in section 5/3. All determinations were performed in triplicate and the following cultures were prepared: control cultures in EBM only; control cultures with 0.42% ethanol; control cultures with PHA at 0.63  $\mu$ l/ml; control cultures with 0.42% ethanol and PHA 0.63  $\mu$ l/ml; and cultures containing each of the three FA studied (dissolved in ethanol) at a concentration of 0.08 mg/ml. The cultures containing FA were allowed to interact with the cells for one hour in the dark at room temperature before being stimulated with PHA (0.63  $\mu$ l/ml) and thereafter incubated for 48 hours, with  $^3\text{H}$ -thymidine added after 44 hours. Results in all cases were expressed as mean cpm  $\times 10^3 \pm$  S.E.M.

The results of this experiment are summarised in figure 5/6. The exact values from the various culture groups were as follows:

Control cultures in EBM :  $0.29 \pm 0.02$

Control cultures in EBM with ethanol :  $0.27 \pm 0.03$

Control cultures with PHA :  $7.7 \pm 2.1$

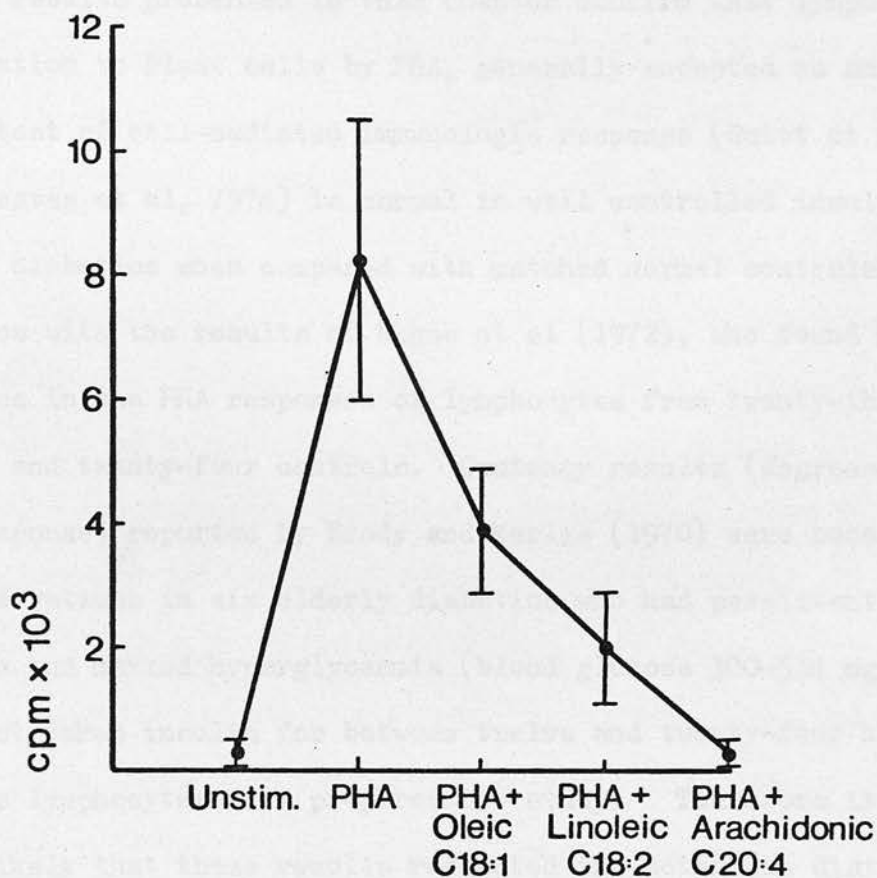
Control cultures with PHA and ethanol :  $8.3 \pm 2.2$

Cultures with oleic acid :  $3.9 \pm 1$

Cultures with linoleic acid :  $2.0 \pm 0.9$

Cultures with arachidonic acid :  $0.3 \pm 0.01$

Statistical analysis confirmed a highly significant suppression of PHA-induced lymphocyte transformation in all cultures to which FA had been added ( $p < 0.01$  by Wilcoxon's test). Ethanol alone had no effect on transformation. The extent of suppression was related to the degree of unsaturation of the FA and was most marked with the PUFA/

FIGURE 5/6

Effects of various fatty acids (in a concentration of 80  $\mu\text{g}/\text{ml}$  culture) on PHA-induced transformation of lymphocytes from twelve normal subjects. Control cultures (Unstim.) contain no PHA; all other cultures contain PHA at 0.63  $\mu\text{l}/\text{ml}$ . All fatty acids induce highly significant ( $p < 0.01$ ) depression of lymphocyte transformation, most marked with arachidonic acid.

Results expressed as in Figure 5/5.

From MacGuish (1976)

PUFA linoleic and arachidonic acid; transformation was completely suppressed in cultures containing the latter PUFA, and differed significantly ( $p < 0.01$ ) from all other cultures containing PHA.

#### 5/10 Discussion of results

The results presented in this chapter confirm that lymphocyte transformation to blast cells by PHA, generally accepted as an in vitro test of cell-mediated immunologic response (Roitt et al, 1969; Greaves et al, 1974) is normal in well controlled insulin-dependent diabetics when compared with matched normal controls. This agrees with the results of Ragab et al (1972), who found no differences in the PHA responses of lymphocytes from twenty-three diabetics and twenty-four controls. Contrary results (depression of PHA response) reported by Brody and Merlie (1970) were based on their observations in six elderly diabetics who had persistent glycosuria and marked hyperglycaemia (blood glucose 300-514 mg/dl) and had not taken insulin for between twelve and twenty-four hours before the lymphocytes were prepared for study. Therefore it appears likely that these results reflected the metabolic disturbance of poorly controlled diabetes, rather than any inherent immunologic abnormality, and the present findings in poorly-controlled diabetic subjects agree with this hypothesis.

Further support for the view that depressed PHA response in diabetics is due to metabolic abnormality is provided by the finding of normal numbers of circulating T and B lymphocytes in diabetics, irrespective of whether they were well or poorly controlled at the time of study. Since PHA is considered mainly to test the function of the T-cell population (Roitt et al, 1969; Greaves et al, 1974), it/

it would be surprising to find a depression of PHA response in subjects with normal numbers of T cells, unless the serum itself contains factors inhibiting lymphocyte transformation. The presence of inhibitory factors is already recorded in diseases such as breast cancer (Whittaker et al, 1971), multiple sclerosis (Knowles et al, 1968) and active syphilis (Levene et al, 1969); from the results obtained in the present studies, it appears that poorly-controlled insulin-dependent diabetics exhibit a similar phenomenon.

What is the nature of the inhibitory factor(s) in diabetic serum? Clearly they are transient, and related to poor control, since lymphocyte transformation is normal in well controlled diabetics. Poorly controlled diabetes is characterised by hyperglycaemia, hyperlipidaemia (especially by mobilisation of FFA in insulin-deficient patients) and (eventually) by ketoacidosis (Alberti and Hockaday, 1977; Dunn, 1982). It seems unlikely that metabolic acidosis per se is the major factor inhibiting lymphocyte transformation since many of the poorly controlled patients studied in the present series showed no significant disturbance of acid-base balance, and mobilisation of FFA must always precede the production of ketone bodies by the liver. On the other hand, the experiments described in sections 5/8 and 5/9 would suggest that hyperglycaemia and FFA are both potent inhibitors of lymphocyte transformation. Of these two influences, it appears that hyperglycaemia plays a lesser part and is probably only of importance when the blood glucose concentration exceeds 300 mg/dl. However FFA causes marked depression of lymphocyte transformation, the depressant effect being especially marked with polyunsaturated fatty acids. Thus it is tempting/



tempting to speculate that the effects of high levels of blood glucose and FFA combine in the poorly controlled diabetic to produce a state of transient immunologic abnormality which may have practical clinical implications in rendering the patient more susceptible to infection. Infections - both viral and bacterial - are identified as the single most important factor in the majority of patients with diabetic ketoacidosis (MacCuish, 1977) and it is usually assumed that the infection has been responsible for the metabolic decompensation. The studies presented in this chapter might support an opposite view, i.e. that poorly controlled diabetes in itself induces a state of temporary immunodeficiency, at least of the cell-mediated limb of the immune system, which makes the patient peculiarly vulnerable to intercurrent infection. With regard to other body defence mechanisms in the same context, Bagdade (1976) has examined granulocyte function in diabetes and has concluded that normal chemotactic, phagocytic and intracellular microbicidal functions of polymorph leucocytes are also largely dependent on good metabolic control. The corollary of all these observations would be that every effort should be made to attain and maintain good metabolic control in insulin-dependent diabetics, and thereby avoid the immunologic abnormalities which may lead to the development of clinically significant infections.

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Parts of this work have already been published: (MacCuish et al, 1974c; MacCuish and Irvine, 1975; MacCuish, 1976).

## CHAPTER SIX

### STUDIES OF THE PREVALENCE OF AUTOANTIBODIES TO NONPANCREATIC ANTIGENS IN DIABETIC AND NONDIABETIC POPULATIONS

#### 6/1 Introduction and purpose of studies

It will be apparent from the General Introduction (section 1/4) that many studies of autoantibody prevalence in diabetic and non-diabetic populations have already been undertaken. However it was felt that some of the findings reported in these studies, and the hypotheses constructed therefrom, justified additional and critical evaluation. The investigations reported in this chapter were therefore undertaken with the following specific objectives:

- a) To examine the differences (if any) in the prevalence of serum autoantibodies between a diabetic and nondiabetic population; and to relate the differences to the age, sex and antidiabetic therapy in the various diabetic subpopulations.
- b) To examine the disparate claims by Nerup and Binder (1973) and Whittingham et al (1971) that increasing duration of insulin-dependent diabetes was associated respectively with increasing or decreasing autoantibody prevalence.
- c) To ascertain whether a population of diabetics with evidence of the specific vascular lesions of the disease (i.e. diabetic microangiopathy) showed a differing autoantibody prevalence from a diabetic population with no evidence of such lesions.

#### 6/2 Selection of diabetic and nondiabetic populations for study

A total of four hundred diabetics were studied, subdivided into the following groups:

##### Insulin-dependent diabetics with microangiopathy

This group comprised one hundred and fifty patients (half being women/

women, half men) with clinical evidence of diabetic microangiopathy. In eighty-five there was evidence of retinopathy alone, the ocular lesions consisting of a background type of retinopathy with microaneurysms, blob haemorrhages and exudates; venous retinopathy, with or without associated haemorrhage; and primary or secondary proliferative retinopathy (neovascularisation). In many of these patients, especially the younger subjects, a mixed type of retinopathy was present in which some or all of the above elements were combined. In several the retinopathy had been treated as appropriate with photocoagulation, laser-beam therapy or the use of the drug clofibrate (Atromid-S). None of the patients had clinical evidence of diabetic nephropathy and none had measurable proteinuria or an elevated blood urea.

The remaining sixty-five patients in this group had more widespread clinical evidence of diabetic microangiopathy. All had clinical signs of oculorenal pathology: the ocular lesions were as described above while the renal lesions consisted of proteinuria, nephrotic syndrome or progressive uraemia. In several cases the diagnosis of diabetic nephropathy had been histologically confirmed by renal biopsy. The ocular lesions tended to be especially severe in these patients with diffuse microangiopathy, and ten of the sixty-five had previously undergone pituitary ablation (Cullen et al, 1971) in an effort to arrest the progression of their retinopathy. Two patients were being treated by intermittent haemodialysis for end-stage renal failure due to diabetic nephropathy and several had evidence of autonomic neuropathy, manifest by abnormal vasomotor responses and gastrointestinal disturbance.

The/

The age and sex distribution of the patients in this group is shown in detail in tables 6/1 and 6/2.

#### Insulin-dependent diabetics without clinical microangiopathy

This group again consisted of one hundred and fifty patients, matched exactly for age and sex with respect to the first group, and as closely as possible for duration of insulin-dependent diabetes. No patient in this group had clinical evidence of diabetic retinopathy or nephropathy, none had measurable proteinuria and all had a normal blood urea. No renal biopsies had however been performed in this group.

The age and sex distribution of the patients in this group is shown in detail in tables 6/1 and 6/2.

#### Insulin-independent diabetics

There were one hundred patients in this group; in forty-one their disorder was controlled by dietary restriction of carbohydrate alone while the remainder were controlled by diet plus an oral hypoglycaemic agent (sulphonylurea or biguanide). No conscious effort was made to exclude patients with specific complications, and thirteen had evidence of a mild background diabetic retinopathy. However no patient had severe ocular or renal disease.

The age and sex distribution of the patients in this subgroup is shown in detail in tables 6/1 and 6/2.

The age of onset and the duration of clinical diabetes were noted for each patient. Care was also taken to exclude any diabetic with known coexisting autoimmune disease or endocrinopathy, or any personal history of those disorders.

#### Control subjects

There/



TABLE 6/1

Analysis of 400 diabetic patients studied,  
according to antidiabetic treatment required

Treatment	Age at time of study			
	10-39 years		40-69 years	
	Male	Female	Male	Female
Diet only	4	10	12	15
Oral hypoglycaemics	11	15	18	15
Insulin/angiopathy+	35	35	40	40
Insulin/angiopathy-	35	35	40	40
Totals	85	95	110	110

Angiopathy+ = patients with specific diabetic  
microvascular lesions

Angiopathy- = patients without specific diabetic  
microvascular lesions.

(see text)

TABLE 6/2

Age and sex distribution of  
diabetic patients and control subjects

Age by decades						
	2nd	3rd	4th	5th	6th	7th
Females	25	35	35	42	41	27
Males	18	30	37	39	38	33
Totals	43	65	72	81	79	60

205 females/195 males  
(Total 400)

Age and sex distribution of  
insulin-dependent diabetic patients and controls

Age by decades							
	2nd	3rd	4th	5th	6th	7th	
Females	21	28	21	24	29	27	150 females/150 males (Total 300)
Males	20	29	21	34	25	21	
Totals	41	57	42	58	54	48	

Age and sex distribution of  
insulin-independent diabetic patients and controls

Age by decades						
	2nd	3rd	4th	5th	6th	7th
Females	1	5	19	13	10	7
Males	1	3	11	10	15	5
Totals	2	8	30	23	25	12

105 females/95 males  
(Total 100)

There were a total of four hundred control subjects, matched for age and sex with the diabetics in all the above subgroups. Most of the controls were healthy volunteers, consisting of laboratory or hospital personnel; many of the middle-aged controls were University servitors and were obtained with the kind assistance of Dr. E.W. Barnes. Hospital outpatients were used in several instances as controls for teenage and elderly diabetics, but none of the controls obtained from this or any other source were known to have endocrine or autoimmune disease.

The age and sex distribution of the control subjects, which is identical to that of the diabetics, is shown in detail in table 6/2.

### 6/3 Methods used for the detection of autoantibodies

Serum samples were obtained from each patient and control, stored at  $-20^{\circ}\text{C}$ , and tested for the presence of the following antibodies:

Antibody to thyroglobulin using the tanned-cell haemagglutination technique (Fulthorpe et al, 1961). The reagents were supplied in kit form (Wellcome Reagents Ltd.) and a detailed description of the technique used is given in Appendix I/3 of this thesis. The test was routinely performed in serum dilutions of 1:5, 1:25, 1:2500 and 1:25000; a titre of greater than or equal to 1:25 was taken as significant.

Antibody to thyroid cytoplasm using the indirect immunofluorescence (Coons) technique, a detailed description of which is given in Appendix I/2 of this thesis. The antigen for this test consisted of human thyroid gland, obtained at partial thyroidectomy for thyrotoxicosis. Small blocks of this tissue were snap-frozen, cut into thin (5 micron) sections/

sections on a cryostat and used as tissue substrate.

Antibody to gastric parietal-cell cytoplasm using the indirect immunofluorescence technique. The tissue substrate for this test consisted of human gastric mucosa, obtained from the body of the stomach at partial gastrectomy and prepared as above.

Antibody to mitochondria using the indirect immunofluorescence technique. Rat kidney was used as the tissue substrate in this test, prepared as above. Staining of mitochondria in the renal tubules was particularly noted and sera giving positive reactions in this fashion were regarded as negative for thyroid-cell and gastric-cell antibodies, because of the nonspecific nature of mitochondrial antibodies.

Antibodies to cell nuclei (ANF) using the indirect immunofluorescence technique and examining all three tissues above (thyroid, stomach, kidney) for the presence or absence of nuclear staining.

Each serum sample was tested with the three tissue substrates mounted together on the same slide, thus providing a check for tissue-specificity of any antibodies detected.

In addition to these tests, the presence or absence of antibodies to adrenal cortex was established in sera from a proportion of the diabetics (one hundred and twenty-three insulin-dependent patients, fifty-seven insulin-independent patients) and their corresponding nondiabetic controls. The technique used was again the indirect immunofluorescence test, the tissue substrate consisting of human adrenal gland which had been removed as soon as possible after death.

The results of antibody determination in diabetics and controls were/



were compared statistically by using the chi-squared test and employing Yates' correction for small samples where appropriate.

#### 6/4 Results of autoantibody studies

##### 6/4a Overall antibody prevalence in diabetics and controls

Table 6/3 summarises the overall differences in antibody prevalence between diabetics and controls. The prevalence of antibodies to thyroglobulin was marginally higher in male diabetics and in patients of either sex aged under forty years ( $p < 0.05$  in both subgroups). There were however no significant differences when female diabetics and diabetics of either sex aged forty years or over were compared with their corresponding controls, nor did the prevalence of thyroglobulin antibody in the whole diabetic group differ significantly from that in the control group. Likewise there were no significant differences in the prevalence of antibodies against cell nuclei or mitochondria between diabetics and controls (overall prevalence 1% for each antibody in both groups).

In contrast there was a highly significant increase in the prevalence of antibodies to both thyroid cytoplasm and gastric parietal-cell cytoplasm in diabetics as compared to controls: the difference was highly significant when the overall groups were compared ( $p < 0.001$ ) for each antibody) and when subgroups of subjects according to sex and age were considered ( $p < 0.001$  -  $p < 0.005$  in all subgroups; table 6/3).

As figure 6/1 shows, a rising prevalence of all three antibodies (thyroglobulin, thyroid cytoplasm, gastric parietal-cell cytoplasm) was observed with advancing age in diabetics and in controls. In the case of thyroglobulin, antibody prevalence became identical in diabetics/

TABLE 6/3

Autoantibody prevalence in controls and diabetics,  
according to sex and age

Group (Number in group)	Number* positive for antibody to:				
	T/G	T/C	PCA	ANF	Mito.
Female controls (205)	23	26	24		
Female diabetics (205)	26(NS)	52(<0.005)	51(<0.005)		
Male controls (195)	7	8	8		
Male diabetics (195)	11(<0.05)	17(<0.005)	23(<0.001)		
Controls aged 10-39 yrs (180)	9	8	7		
Diabetics aged 10-39 yrs (180)	14(<0.05)	27(<0.001)	29(<0.001)		
Controls aged 40-69 yrs (220)	21	26	25		
Diabetics aged 40-69 yrs (220)	23(NS)	42(<0.005)	45(<0.005)		
All control subjects (400)	30	34	32	5	5
All diabetic subjects (400)	37(NS)	69(<0.001)	74(<0.001)	6(NS)	4(NS)

T/G = antibody to thyroglobulin

T/C = antibody to thyroid cytoplasm

PCA = antibody to gastric parietal-cell cytoplasm

ANF = antibody to cell nuclei

Mito. = antibody to mitochondria

\*Figures in parentheses = statistical significance of the prevalence of the antibodies in subgroups of diabetics compared to that in matched controls.

Statistical analysis by the chi-squared test. NS = not significant.

FIGURE 1

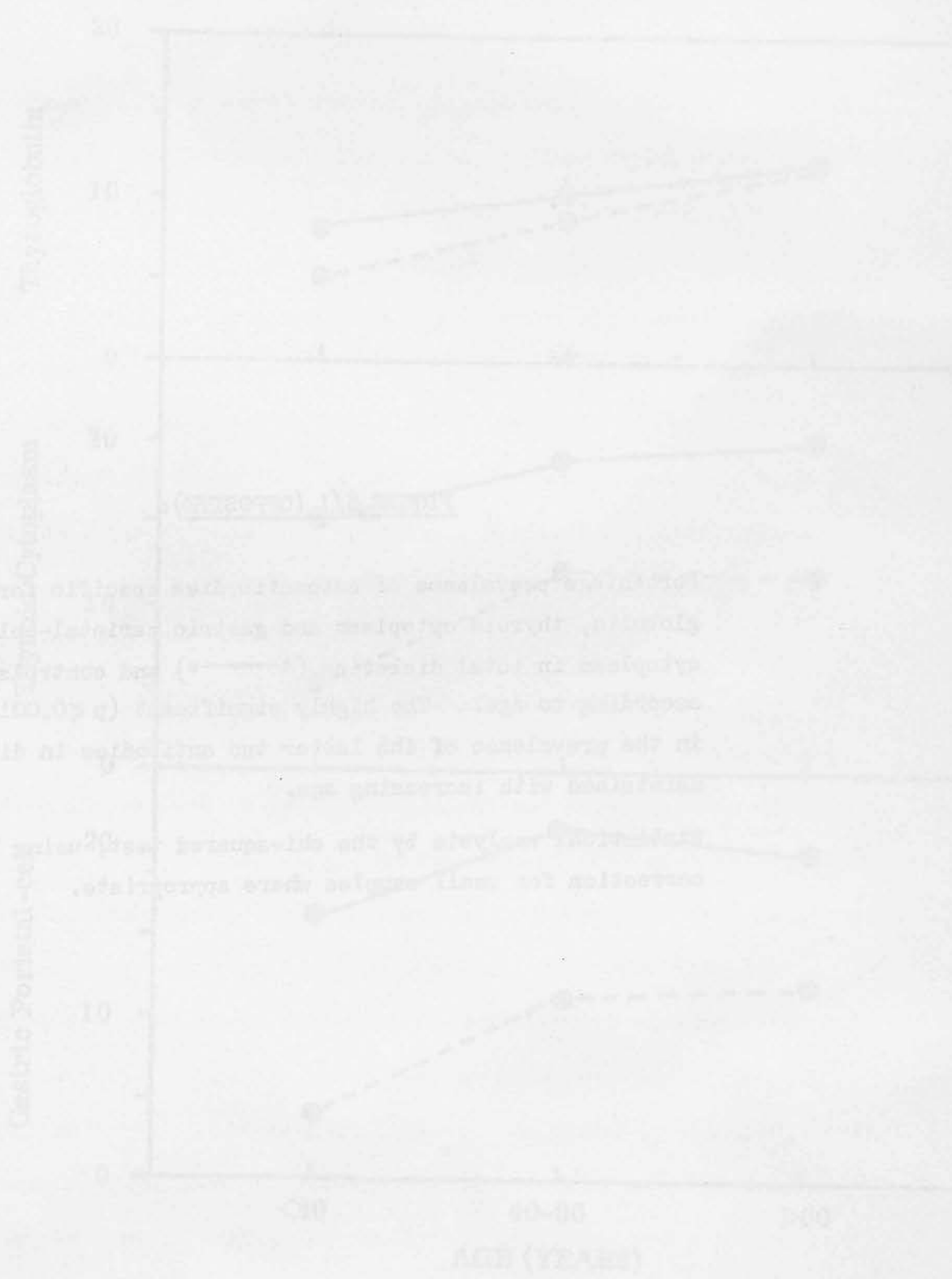


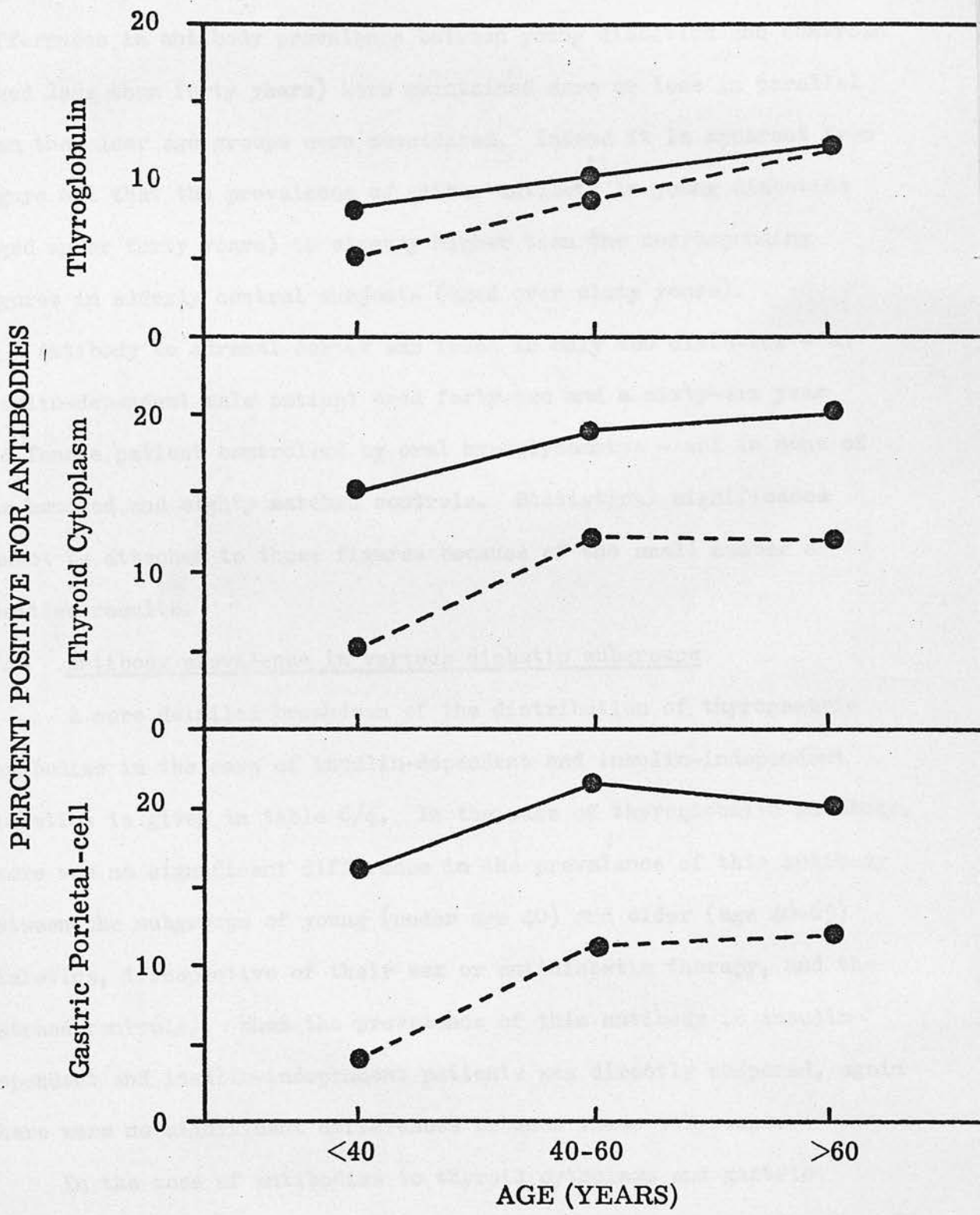
FIGURE 6/1 (OPPOSITE):

Percentage prevalence of autoantibodies specific for thyroglobulin, thyroid cytoplasm and gastric parietal-cell cytoplasm in total diabetics (●—●) and controls (●- - -●) according to age. The highly significant ( $p < 0.001$ ) differences in the prevalence of the latter two antibodies in diabetics are maintained with increasing age.

Statistical analysis by the chi-squared test, using Yates' correction for small samples where appropriate.



FIGURE 6/1



diabetics and controls aged over sixty years. In the case of antibodies to thyroid cytoplasm and gastric parietal-cell cytoplasm, the differences in antibody prevalence between young diabetics and controls (aged less than forty years) were maintained more or less in parallel when the older age groups were considered. Indeed it is apparent from figure 6/1 that the prevalence of either antibody in young diabetics (aged under forty years) is already higher than the corresponding figures in elderly control subjects (aged over sixty years).

Antibody to adrenal cortex was found in only two diabetics - an insulin-dependent male patient aged forty-two and a sixty-six year old female patient controlled by oral hypoglycaemics - and in none of one hundred and eighty matched controls. Statistical significance cannot be attached to these figures because of the small number of positive results.

#### 6/4b Antibody prevalence in various diabetic subgroups

A more detailed breakdown of the distribution of thyrogastic antibodies in the sera of insulin-dependent and insulin-independent diabetics is given in table 6/4. In the case of thyroglobulin antibody, there was no significant difference in the prevalence of this antibody between the subgroups of young (under age 40) and older (age 40-69) diabetics, irrespective of their sex or antidiabetic therapy, and the matched controls. When the prevalence of this antibody in insulin-dependent and insulin-independent patients was directly compared, again there were no significant differences between these subgroups.

In the case of antibodies to thyroid cytoplasm and gastric parietal-cell cytoplasm, it is apparent that the highly significant increase in the prevalence of both antibodies in diabetes is mainly accounted/

The distribution of positive tests for thyrogastric antibodies  
to diabetics according to age, sex and treatment

Group	Controls		Diabetics*			
	Female	Male	Insulin-dependent		Insulin-independent	
			Female	Male	Female	Male
<u>Aged 10-39 years:</u>						
No. positive for antibody to:						
Thyroglobulin	7	2	7(NS)	3(NS)	3(NS)	1(NS)
Thyroid cytoplasm	6	2	18(<0.001)	5(<0.001)	3(<0.05)	1(NS)
Gastric parietal-cell cytoplasm	6	1	19(<0.001)	7(<0.001)	2(NS)	1(NS)
Total no. of subjects	95	85	70	70	25	15
<u>Aged 40-69 years:</u>						
No. positive for antibody to:						
Thyroglobulin	16	5	12(NS)	5(NS)	4(NS)	2(NS)
Thyroid cytoplasm	20	6	25(<0.005)	9(<0.005)	6(<0.05)	2(NS)
Gastric parietal-cell cytoplasm	18	7	27(<0.001)	12(<0.001)	3(NS)	3(<0.05)
Total no. of subjects	110	110	80	80	30	30

\*Figures in parentheses = statistical significance of antibody prevalence in subgroups of diabetics compared to that in matched controls.

Statistical analysis by the chi-squared test, using Yates' correction for small samples.  
NS = not significant.

accounted for by insulin-dependent patients of either sex, irrespective of age ( $p < 0.001$  -  $p < 0.005$  in all subgroups). The differences in antibody prevalence between insulin-independent patients and controls were much less striking, only reaching weak statistical significance ( $p < 0.05$ ) in three of the eight subgroups indicated in table 6/4.

When the prevalence of thyroid-cell antibody in insulin-dependent and insulin-independent diabetics was directly compared, there was a significant increase in insulin-dependent females of any age ( $p < 0.005$ ) but no significant differences between insulin-dependent and insulin-independent males. Similar significance was recorded for the differing prevalence of gastric parietal-cell antibody in insulin-dependent and insulin-independent females, while the higher prevalence of this antibody in insulin-dependent males also reached weak statistical significance ( $p < 0.05$ ).

#### 6/4c Comparison of antibody prevalence in insulin-dependent diabetics with and without clinical microangiopathy

The prevalence of thyrogastric antibodies in insulin-dependent diabetics with clinical microangiopathy is compared in table 6/5 with the figures in age- and sex-matched diabetics who had no clinical evidence of such lesions. No significant differences between the groups were apparent.

Antibody prevalence as related to duration of insulin-dependent diabetics in these two groups is compared in table 6/6: again there were virtually no differences between patients with and without microangiopathy, although the prevalence of gastric parietal-cell antibody was marginally higher ( $p < 0.05$ ) in male patients with microangiopathy who had had the disorder for less than five years. However the number of subjects in this subgroup is small and it is probable that the difference/



TABLE 6/5

The distribution of positive tests for thyrogastric autoantibodies in insulin-dependent diabetics, according to age and the presence or absence of specific diabetic complications

Group	Insulin-dependent diabetics*	
	Angiopathy-	Angiopathy+
<u>Aged 10-39 years:</u>		
No. positive for antibody to:		
Thyroglobulin	8	6(NS)
Thyroid cytoplasm	12	15(NS)
Gastric parietal-cell cytoplasm	13	16(NS)
Total no. of subjects	70	70
<u>Aged 40-69 years:</u>		
No. positive for antibody to:		
Thyroglobulin	10	13(NS)
Thyroid cytoplasm	19	23(NS)
Gastric parietal-cell cytoplasm	20	25(NS)
Total no. of subjects	80	80

Angiopathy+ = patients with clinical evidence of specific complications.

Angiopathy- = patients with no clinical evidence of specific complications.

(See text)

\*Figures in parentheses = statistical significance of the prevalence of antibodies in subgroups of diabetics with microangiopathy compared to that in age- and sex-matched patients without microangiopathy.

Statistical analysis by the chi-squared test, using Yates' correction.

NS = not significant.

TABLE 6/6

The distribution of positive tests for thyrogastric autoantibodies  
in insulin-dependent diabetics, according to duration of disease  
and the presence or absence of specific diabetic complications

Group	Insulin-dependent diabetics*	
	Angiopathy-	Angiopathy+
<u>Duration of diabetes &lt;5 years:</u>		
No. positive for antibody to:		
Thyroglobulin	7	6(NS)
Thyroid cytoplasm	16	12(NS)
Gastric parietal-cell cytoplasm	15	16( 0.05)
Total no. of subjects	73	45
<u>Duration of diabetes 5-10 years:</u>		
No. positive for antibody to:		
Thyroglobulin	8	6(NS)
Thyroid cytoplasm	11	16(NS)
Gastric parietal-cell cytoplasm	13	15(NS)
Total no. of subjects	58	66
<u>Duration of diabetes &gt;10 years:</u>		
No. positive for antibody to:		
Thyroglobulin	3	7(NS)
Thyroid cytoplasm	4	10(NS)
Gastric parietal-cell cytoplasm	5	10(NS)
Total no. of subjects	19	39

Explanation as for table 6/5.

\*Figures in parentheses = statistical significance of the prevalence of antibodies in subgroups of diabetics with microangiopathy compared to that in the corresponding subgroups of patients without microangiopathy.

Statistical analysis as for table 6/5.

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difference would not be sustained if a larger number of patients had been studied.

If the data in table 6/6 is analysed vertically, i.e. with respect to lengthening duration of diabetes within each group, there is no suggestion of either an increase or decrease in antibody prevalence with increasing duration of clinical diabetes in patients with and without microangiopathy.

## 6/5 Discussion of results

The observed differences in antibody prevalence between the diabetic and nondiabetic populations in this study are in broad agreement with the findings reported by earlier investigators and summarised in the introduction to this thesis (section 1/4; tables 1/5, 1/6). The overall prevalence of antibody to thyroglobulin is virtually identical in both groups, while there is a striking increase in the prevalence of antibodies to both thyroid cell cytoplasm and gastric parietal-cell cytoplasm in diabetic patients. A detailed breakdown of the distribution of these antibodies in diabetic subgroups has shown clearly that thyrogastric antibodies occur far more commonly in insulin-dependent than insulin-independent patients and are especially common in women. The figures for insulin-independent diabetics are in contrast much closer to those for the control population. Antibodies which are tissue-specific rather than organ-specific (ANF or mitochondrial antibody) are found with equal frequency in diabetics and nondiabetics, and have no clinical significance outwith the context of connective-tissue disorders or biliary disease. Antibodies to adrenal cortex are found only rarely in diabetics but virtually never in nondiabetics, except in the context of idiopathic Addison's/

Addison's disease.

These findings are not novel but are incidental to the main purpose of the present study, which was designed with two specific objectives. The first - to examine antibody prevalence in relation to the duration of insulin-dependent diabetes - was prompted by the conclusions reached by the two groups of workers who had previously investigated this aspect of immunity in diabetes. Nerup and Binder (1973) studied autoantibodies in the sera of 132 diabetics and 128 controls: in the subgroup of 44 patients who had been diabetic for more than ten years, they found a significant increase in thyrogastric antibody prevalence compared to the matched control subgroup. The increase was only weakly significant ( $p < 0.05$ ) but was taken as evidence that increasing duration of 'juvenile-onset' diabetes was associated with increasing autoantibody prevalence. However it is difficult to see how this conclusion could be sustained as the same authors proceeded to compare thyrogastric antibody prevalence directly in their subgroups of newly-diagnosed diabetics (duration less than one year) and longstanding diabetics (duration more than ten years) and found no differences between these groups. Furthermore, Nerup and Binder detected no differences in antibody prevalence between 'juvenile-onset' (insulin-dependent) and 'maturity-onset' (insulin-independent) patients, which is in marked contrast to the findings in the much larger series of Ungar et al (1968) and Irvine et al (1970). Somewhat different results were reported by Whittingham et al (1971) in a series of 400 diabetics and matched controls: these authors described a significant decrease in thyrogastric antibody prevalence amongst/



amongst ageing diabetics. The decrease was especially notable in insulin-dependent patients, predominantly women, of more than twenty years' standing, and Whittingham et al proffered the suggestion that the phenomenon was accounted for by the premature death of diabetics with positive thyrogastric antibodies. However no statistical values were offered in support of the decreasing antibody prevalence, which at best seemed only weakly significant.

The present study has not confirmed the findings of Nerup and Binder or of Whittingham and her colleagues. On the contrary it was found that high prevalence of thyrogastric antibodies was invariably associated with insulin-dependent diabetes, and that the differences between diabetics and controls were quite independent of the age of the patients (figure 6/1; table 6/4) or the duration of insulin-dependent diabetes (table 6/6). The frequent possession of these antibodies seems to be a feature of insulin-dependent diabetes at the very onset of clinical disease, and any increase thereafter merely parallels the anticipated increase with advancing age which is seen in the normal population.

The second objective was to examine any possible relationship between antibody status and the presence of significant microvascular disease in insulin-dependent diabetes. No previous study of this nature had been undertaken, and it was tempting to speculate that autoantibodies might have been found more commonly in patients with severe microangiopathy: the grossly abnormal basement membrane of affected diabetic blood vessels, coupled with ischaemic lesions in affected organs, might have allowed the passage into the circulation of material which would elicit a humoral antibody response. Again, if/

if the findings of Whittingham et al had been sustained, the presence of autoantibodies might conceivably have been associated with particularly severe microangiopathy, as a potent cause of premature death amongst longstanding diabetics. Neither speculation can be justified, however, as the results have clearly shown (tables 6/5, 6/6) that the presence or absence of significant (i.e. clinically detectable) diabetic microangiopathy is not associated with any particular increase or decrease in autoantibody prevalence, irrespective of the age of the patient or the duration of diabetes.

In conclusion it can therefore be said that the studies presented in this chapter have provided further support for the belief that autoantibody formation is an integral part of the diathesis of insulin-dependent diabetes, and is independent of the age of onset, the duration of treatment or the specific complications of the disease. It is not possible to estimate how frequently the presence of antibodies in these patients is the precursor of overt autoimmune disease but the strong clinical associations of diabetes with thyroid, gastric and adrenal autoimmunity have already been commented upon.

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Part of this work has already been published (MacGuish and Irvine, 1975).

## CHAPTER SEVEN

### ENVIRONMENTAL AND GENETIC FACTORS IN THE POSSIBLE GENESIS OF AUTOIMMUNE DIABETES MELLITUS: SUMMARY AND CONCLUSIONS

#### 7/1 Introduction

It is believed that the studies contained in the preceding chapters, in which a variety of humoral and cellular autoimmune phenomena have been identified in certain cases of diabetes mellitus, lend credibility to the concept that diabetes may sometimes be a true organ-specific autoimmune disease. When this concept is considered, however, it is obligatory to examine two difficult questions. First, what kind of factor might trigger off an autoimmune process leading to endocrine pancreatic failure in a susceptible subject? Second, what peculiarity or defect in that subject's immune apparatus might make him or her susceptible to that stimulus? The answers to both questions are, naturally, obscure; nonetheless it is important to examine the available evidence which has suggested that viral infection may sometimes initiate the onset of diabetes in man and indicated that persons with a general susceptibility to autoimmune processes (including diabetes) may be identifiable by histocompatibility (HLA) studies.

#### 7/2 Viral infection and autoimmunity in diabetes mellitus

The suggestion that viral infection might be aetiologically related to diabetes mellitus was apparently first offered by Harris (1899), based on his observation that an attack of mumps might be closely followed by the development of clinical diabetes. Since then it has been established both clinically and experimentally that viral infection can invade the pancreas of many animal species. The induction/

induction of carbohydrate intolerance by viral inoculation of the experimental animal was first accomplished by Craighead and McLane (1968), who inoculated mice with the M variant of the encephalomyocarditis (EMC) virus to produce a disease which clinically and pathologically resembled human, abrupt-onset diabetes with insulinitis. Numerous subsequent investigators have since documented the ability of variants of the EMC virus, of group B Cocksackie viruses and of reovirus to induce a diabetes-like syndrome in rodents (Craighead and Steinke, 1971; Muntefering et al, 1971; Wellman et al, 1972; Coleman et al, 1973; Craighead, 1975; Notkins, 1977; Onodera et al, 1978; Yoon et al, 1978; table 1/10). From these and other studies it has become clear that differing strains or variants of these viruses can cause either a diffuse pancreatitis or more specific lesions of the endocrine pancreas; thus the inoculation of the E variant of the EMC virus and Cocksackie B3 virus produce a generalised pancreatitis without specific insular damage or the development of diabetes, while the M variant of EMC virus, Cocksackie B4 virus and reovirus type 3 induce only a mild exocrine pancreatitis but a severe, rapidly-evolving insulinitis which is invariable accompanied by diabetes. These latter, 'specific' insulinitis-producing viruses seem to produce a typical series of pancreatic changes: within forty-eight hours of their inoculation, many islets show interstitial oedema and marginal or central necrosis of cells. On the third and fourth days, there is extensive and total necrosis of some islets, both alpha and beta cells being involved, and macrophages begin to infiltrate between the islet cells at this time. Between the third and the fifteenth day, but most commonly after about a week, poorly-developed inflammatory infiltrates of the pancreas are seen/



seen, and the islets are infiltrated by mononuclear round cells and a few polymorphs. Marked degeneration of the surviving beta cells is evident. The lesions are always patchy, many islets being initially normal on light microscopy, but latterly these ostensibly unharmed islets exhibit advancing beta cell degranulation. The extent of islet-cell damage seems to be partly dependent on the metabolic state of the animal at the time of inoculation; mice who have been pre-treated with steroids or rendered obese by gold-thi-glucose injection show particularly severe lesions (Craighead, 1966), suggesting that beta cells which are most active metabolically are also the most vulnerable to damage by viral invasion. Moreover, reference has already been made (chapter 1/6) to the fact that only certain strains of mice possess a genetic susceptibility to viral infection which results in the development of insulitis and diabetes, and that this susceptibility is associated with the possession by susceptible animals of certain immune response genes within the major (H-2) murine histocompatibility complex.

In man, evidence to support a role of viral infection in the aetiology of diabetes has been confined to insulin-dependent (Type 1) diabetes, where the clinical onset of disease, with its abrupt symptoms, provides a convenient temporal point for the epidemiologist to relate to associated clinical or pathological phenomena. Indirect evidence to associate viral infections with the acute onset of IDDM includes data on seasonal variation of disease, and the association of common childhood viral infections with the subsequent onset of diabetes. The earliest epidemiological studies were those of Gamble and Taylor (1969) who first described an increased incidence of cases of IDDM in Britain/

Britain during the autumn and winter seasons. Several other large studies have subsequently confirmed these observations: for example, the estimated time of onset of IDDM in 2511 children living in four different American States followed a similar seasonal pattern (peaks in the late summer, early autumn, winter months) and a study of the onset of IDDM in 851 Australian children showed peaks of onset in autumn and winter months (MacMillan et al, 1977; Fleegler et al, 1979). All these studies suffer from the common disadvantages of being retrospective, depending on information from parental recall or clinical records and based on the assumption that there is a short interval between infection and onset of clinical diabetes.

With regard to viral diseases which might be causally implicated in the induction of diabetes in man, the specific infections reported to be associated with the onset of IDDM include mumps, rubella, Coxsackie virus, infectious hepatitis, infectious mononucleosis and cytomegalovirus (Craighead, 1981). Most of these reports are based on observations of one or at most a few cases: the exception is the work of Gamble and colleagues, who correlated cases of IDDM in autumn and winter with the seasonal prevalence data for Coxsackie type B4 viral infections. By retrospective serological testing, they were able to show that sera from IDDM patients with clinical disease duration of less than three months contained significantly higher antibody titres to Coxsackie B4 than the sera of nondiabetic controls or IDDM with longstanding duration of disease (Gamble et al, 1969). These observations are interesting but require to be confirmed, preferably on a prospective basis, and it is curious that later studies by the same authors (Gamble et al, 1973) and others (Baum, 1974; Hierholzer and/

and Farris, 1974; Nelson et al, 1975; Dippe et al, 1975) apparently failed to show any association between Coxsackie (or other) viral infections and the development of IDDM in children and young persons. However, all these studies have suffered from the defect of assuming that IDDM must ensue within a reasonably short time after viral infection and it is quite possible that a long time interval can elapse between the initial viral insult and the ultimate destruction of sufficient beta cells to cause insulin deficiency. Indeed a lengthy time interval between infection and diabetes might be considered to be more likely if the virus is exerting its effects by inducing an immunologic reaction to pancreatic islets, with a gradual destruction of beta cells, rather than causing a direct destructive intracellular infection of beta cells. Support for the hypothesis that a long interval may occur between the viral infection and the appearance of IDDM is given by the study of Sultz et al, who found an increased prevalence of new cases of IDDM in Erie County, New York, several years after the community-wide outbreak of mumps (Sultz et al, 1975). Moreover, pancreatic cytoplasmic islet-cell antibodies have recently been detected in (nondiabetic) children following mumps infection (Helmke et al, 1980) and again this would argue that an immunologic process may be initiated by viral infection but may not lead to the rapid onset of overt IDDM. The studies which have indicated the value of cytoplasmic ICA as a 'marker' for the eventual appearance of IDDM have already been reviewed in chapter two, and it is worth re-emphasising that a long period of apparently normal carbohydrate metabolism may elapse between the detection of ICA and the onset of IDDM (Irvine, Gray and Steel, 1980; Gorsuch et al, 1981; Bottazzo/

Bottazzo et al, 1981).

The possible mechanisms whereby viral infection of the pancreas or other tissue could initiate immunopathological processes have been well reviewed by Freytag (1974). Virus invasion of the host is followed by two differing cell changes, according to the general properties of viruses: the virus either multiplies and matures in one cell and after cytolysis invades other cells, producing spreading infection, or there is a steady-state interaction in which the virus replicates in the host cells, leaving the host structurally intact but producing functional alterations within the infected cells. Either or both of these virally-mediated cell changes can lead to a wide variety of different immunological reactions which probably all have cytotoxic effects. In the specific context of autoimmunity, Freytag (1974) concludes that viral infection can give rise to:

1. New antigenic sites in the protein of cells.
2. Production of cell-specific antibodies and sensitised lymphocytes.
3. A nonspecific acceleration of general immune responses.

By such cellular changes, virus infection may trigger off an autonomous development of chronic inflammatory processes, i.e. autoimmune processes, even though the virus itself has already been overcome by the host. These mechanisms have provided at least the theoretical basis for a relationship between virus infection and human diabetes with insulitis, and this relationship has now been proven in man. Craighead (1974), examining histological material from infants dying with fully documented Coxsackie B virus infections, found changes in the pancreas (fibrosis, interstitial pancreatitis, lymphocyte infiltration/



infiltration of the islets of Langerhans) identical to those described by Gepts (1965) (chapter one) but did not record the presence of diabetes in these infected children. The extensive pathological study of Jenson et al (1980) examined pancreases from 250 children with fatal infections caused by at least fourteen viruses. Lesions in the islets were found in four cases of Coxsackie B viral infection, twenty cases of cytomegalovirus and two cases each of varicella-zoster and congenital rubella. The cases of Coxsackie B infection showed beta cell destruction and acute or chronic inflammatory infiltrates, similar to the observations of Craighead, while typical inclusion bodies were found in cases of cytomegalovirus and varicella-zoster (Jenson et al, 1980).

Recently Yoon et al (1979) described the isolation of Coxsackie virus B4 from the pancreas of a previously healthy ten year old child (with a strong family history of IDDM) who was admitted in diabetic ketoacidosis and died of meningo-encephalitis after eight days in hospital. At autopsy the pancreas showed the classical changes of insulinitis, while the virus isolated therefrom was injected into susceptible mice and induced diabetes with beta cell destruction (Yoon et al, 1979). At present this striking case has provided the most direct evidence for a viral aetiology of IDDM in an individual patient but Coxsackie B5 has been isolated from the faeces of an infant with viral infection who developed diabetes mellitus a few days later (Champsaur et al, 1980) and a child who developed diabetes and died shortly after open-heart surgery was shown to have Coxsackie B4 antigens in her pancreatic islets (which showed insulinitis) and high titres of B4 antibodies in her blood (Gladisch et al, 1976).

It/

It is likely that further examples will be obtained by using modern methods of isolation and culture of viruses from human tissues.

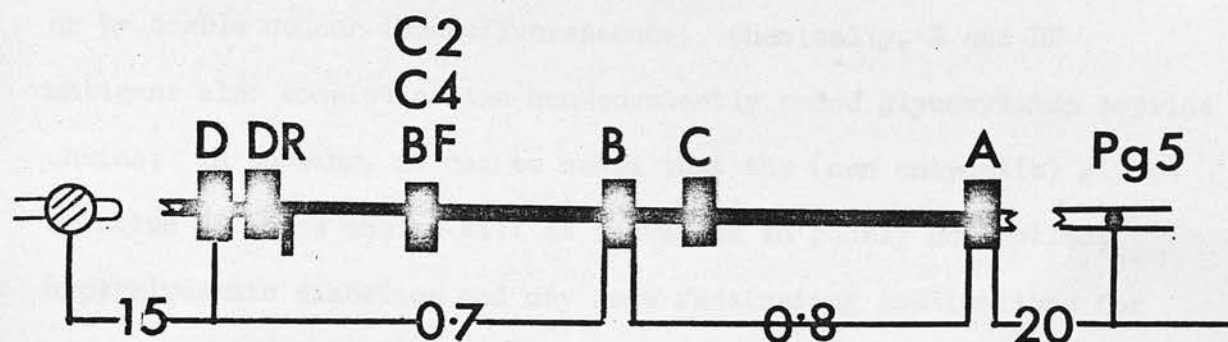
Clarification of a relationship between viral infection and the subsequent development of IDDM has been tantalisingly slow to emerge, despite the epidemiological and pathological studies conducted over the past thirteen years. Viral infections might play only a minor role in catalysing the genetic factors which lead to IDDM and studies (England and Roberts, 1981) which examine the cost effectiveness of antiviral immunisation to prevent IDDM are probably premature. Nevertheless, it has been calculated that if only 15 per cent of IDDM is virally induced, and if a polyvalent vaccine was developed that was only 13 per cent effective, then vaccinating all children at the age of three years would be cost effective and would prevent over one hundred thousand cases of IDDM in the U.S.A. within fifty years (England and Roberts, 1981). At present this kind of exercise is hypothetical, and must await a clearer definition of the role of viruses in IDDM. Thus far, all that can be said is that on epidemiological and pathological grounds, virus infections are a plausible initiating cause of IDDM in persons who have a genetic susceptibility to the disease. Other environmental factors - for example, a widely used pesticide which is similar in structure to streptozotocin - may also be capable of initiating an immunologically-mediated destruction of the islet cells in susceptible individuals and has been implicated in the development of over two hundred cases of IDDM (Karam et al, 1979; see also chapter one, section 1/6); however, the importance of such environmental toxins in the aetiology of IDDM is presently entirely speculative.

### 7/3 Histocompatibility (HLA) antigen typing in diabetes mellitus

If viral infection is indeed proven to be the stimulus (or one of several stimuli) to a series of autoimmune processes which result in diabetes mellitus, histocompatibility (HLA) antigen typing may equally prove to be a means of identifying those subjects whose defective immune apparatus renders them susceptible to these processes. The HLA chromosomal region is situated on the short arm of chromosome 6 and represents approximately one thousandth of the total human genome. The genes coding for HLA antigens A, B, C, D and DR (D-related) occupy closely linked loci in this region, which also is the site for genes coding for C2 and C4 of the classical complement pathway and Bf of the alternative complement pathway (figure 7/1). The HLA system is polymorphic at each locus and there is linkage disequilibrium between the various loci. Linkage disequilibrium means that certain HLA specificities encoded by genes at the different linked loci occur much more frequently together on the same haplotype than would have been predicted on a statistical basis. For example, in Caucasian populations the HLA-B locus antigen B8 occurs together with the HLA-D locus antigen DW3 several times more frequently than expected. The same applies for A1 and B8 or A3 and B7 in North European populations.

The HLA alleles are expressed as cell surface <sup>glyco</sup>proteins:

HLA-A, -B and -C antigens are present on virtually all nucleated cells in the body and are characterised on peripheral blood lymphocytes by using a wide range of antisera in a complement-dependent microlymphocytotoxicity test. Chemically, A, B and C antigens are two-chain glycoproteins which are inserted into the cell membranes. The HLA-D and/

FIGURE 7/1

The HLA system on chromosome 6 (short arm). The major histocompatibility loci (HLA-A, -C, -B, -D and -DR) are shown in black. Other loci are coding for factors C2 and C4 of the classical complement pathway and for Bf of the alternative complement pathway. Approximate distances between the different loci are given in centimorgans,  $\odot$  represents the centromere.



and -DR antigens are much more restricted in expression and are found predominantly on B lymphocytes and macrophages. The former are detected by mixed lymphocyte culture techniques (MLC) and the latter by classical serological methods on separated B lymphocytes or by double colour immunofluorescence. Chemically, D and DR antigens also consist of two non-covalently bound glycosylated peptide chains; in passing, it can be noted that the (non enzymatic) glycosylation of these chains will be increased in poorly controlled, hyperglycaemic diabetics and may have fascinating implications for the genesis of microvascular complications in diabetes.

The earliest study of HLA typing in diabetes was that of Finkelstein et al (1972), who examined a series of forty-four insulin-dependent patients and found no significant alteration in antigen frequency from that in the nondiabetic population. However this study is invalid as the patient population was not only small but racially heterogeneous (Caucasian, Mexican and Negro), and any alterations in antigen frequency would thus have been obscured. In contrast Singal and Blajchman (1973) studying an all-Caucasian population, found a significantly higher frequency (thirty-six per cent) of the antigen B15 in insulin-dependent diabetics when compared with non-insulin-dependent patients and controls (prevalence nine and ten per cent respectively). The latter study was extended in Denmark by Nerup et al (1974b) who reported a significantly higher frequency of antigen B15 and B8 in a series of one hundred and forty-six Caucasian diabetics, and correlated the presence of these antigens with the age of onset of diabetes, the weight of the patient and the type of antidiabetic therapy. Antigen B15 was found in both juvenile and/

and middle aged patients, but usually in association with IDDM, while B8 was found almost exclusively in juvenile-onset, non-obese IDDM. Almost simultaneously a similar positive association between IDDM (Type 1 diabetes) and B8/B15 was established in the U.K. diabetic population (Cudworth and Woodrow, 1975, 1976). Over the next few years, these results were confirmed and extended in multiple population studies: as work proceeded on HLA typing, it has emerged that DW3-DR3 and DW4-DR4 represent the strongest and therefore the primary associations with IDDM (Christy et al, 1979; Cudworth et al, 1979). All the available data now indicates that the associations with HLA-B, -C and -A antigens and complement factors are secondary and due to linkage disequilibrium within the HLA system (Cudworth and Wolf, 1982). The prime importance of DW3, DR3 and DR4 in IDDM has now been demonstrated in populations of Japanese (Sakurami, 1982), black American (Rodey et al, 1979) and Mexican-American IDDM (Zeidler et al, 1982): HLA B8 occurs rarely in all these populations and other HLA locus alleles are found in linkage disequilibrium with the D and DR alleles. In the U.K. between 80 and 95% of Caucasian IDDM populations are now found to possess DR3 or DR4, the relative risks associated with these factors being 7.39 and 9.25 respectively, while possession of both DR3 and DR4 confers a relative risk of IDDM of 14.26 (Cudworth and Wolf, 1982). (The term 'relative risk' is applied to the association between HLA and a particular disease to indicate how much more frequently the disease in question occurs in carriers of a particular antigen as compared with those lacking this antigen: relative risk denotes the strength but not the statistical significance of HLA and disease associations). To date (end of 1982) eighty-three population studies have/

have demonstrated an increased relative risk from the development of IDDM associated with HLA-B8, B15, B18, CW3, DW3, DW4, DR3 and DR4 in Caucasians and DW3, DR3 and DR4 in the Japanese, black American and Mexican-American populations (Albin and Rifkin, 1982; see also Christy et al, 1979 and Gudworth, 1981). By contrast, one remarkable observation found in most (Caucasian) population studies is the extremely low frequency in IDDM of HLA-DR2 and B7, which are in linkage disequilibrium, and it has been postulated that these alleles may exert a 'protective' effect against the development of IDDM (Ludwig et al, 1977; Christy et al, 1979; Gudworth, 1978). The same types of studies have confirmed beyond question that there is no association between NIDDM (Type 2 diabetes) and HLA factors.

Family studies of IDDM in Caucasian populations have amply confirmed the above findings and, by HLA typing both parents and all children in a family, have provided the opportunity to initiate genetic counselling by estimating relative risks in HLA-identical or haplo-identical siblings of an IDDM proband (Gorsuch et al, 1981; Gudworth and Wolf, 1982): for example, it can be demonstrated that an HLA-identical sibling has a 90-times increased risk and a haplo-identical sibling a 37-times increased risk of developing IDDM by the age of 16 years.

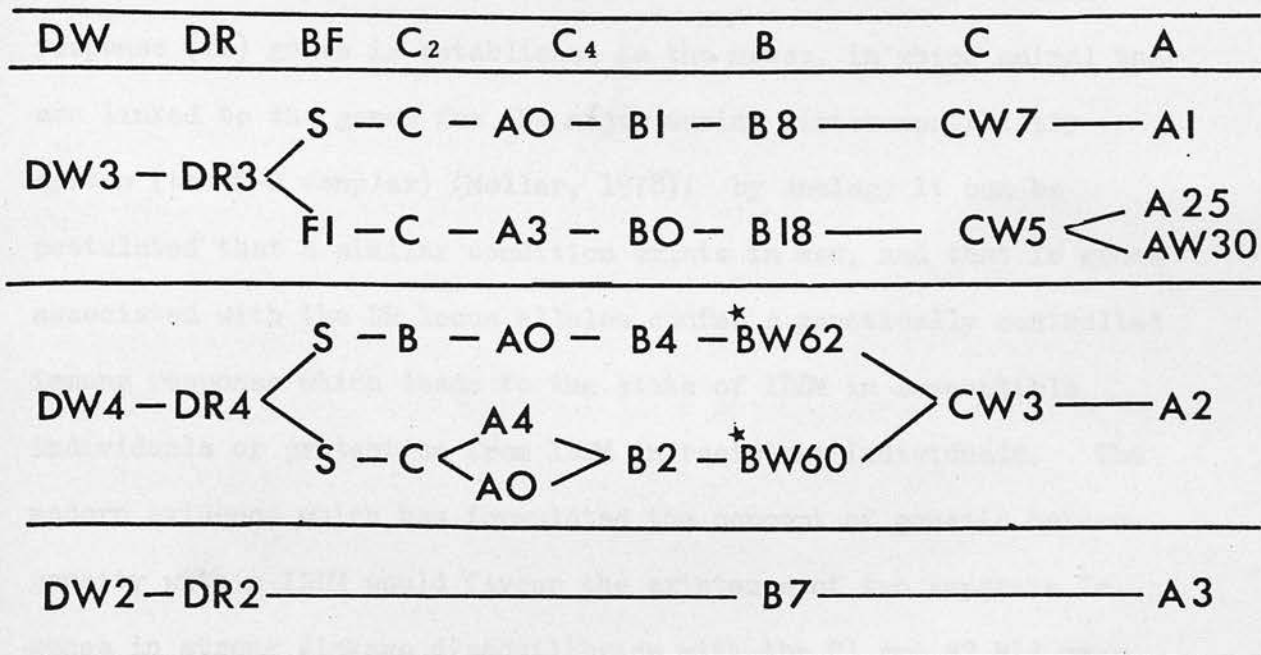
All the extensive population and family studies of HLA in IDDM, paying due regard to the patterns of linkage disequilibrium within HLA, have strongly suggested that genetic heterogeneity occurs within IDDM. It is now possible to define two separate axes of HLA factors, each axis being associated with susceptibility to developing IDDM, and a single axis of HLA factors which confers protection from IDDM (Gudworth/



(Cudworth et al, 1978, 1980). The first susceptibility axis (or S1) is defined by the primary associations of DW3-DR3 and the secondary associations with B8, B18 and A1. The S2 axis is constructed in the same fashion, DW4 and DR4 representing the primary, and B15, B40, CW3 and A2 the secondary associations. By contrast, DW2 and DR2 represent the primary and B7 and A3 the secondary associations which comprise the HLA axis (the R axis) of significant resistance to the risk of developing diabetes. Indeed, the risk of developing diabetes is virtually zero in DW2-DR2 subjects (Nerup et al, 1980). The most up to date (1982) information for each axis is presented in figure 7/2, which takes into account the recent 'splitting' of HLA-B15 into two subgroups (BW62, BW63) and the subdivision of HLA-B40 into BW60 and BW61 (Cudworth and Wolf, 1982).

What biological significance is there in these very striking associations between IDDM and the possession of certain HLA antigens, or indeed the 'protection' from IDDM apparently associated with other HLA antigens? The principle explanations to connect a disease of unknown cause (such as IDDM) with a genetic marker (such as HLA type) are ethnic stratifications, causation or association. The first explanation has already been negated by the population studies referred to above, which found the same HLA antigens associated with IDDM in distinctly different ethnic groups. Causation would imply HLA DR3/DR4 are directly involved in the initial pathogenesis of diabetes: it is certainly possible that these antigens could act as receptors for viruses or hormones, or that their presence on the membrane of the beta cells is necessary to activate the immune recognition system, but all such suggestions are entirely speculative at present. The 'association'/'



FIGURE 7/2

The HLA system in relation to insulin-dependent (Type 1) diabetes mellitus. Two axes of HLA factors are associated with susceptibility and one with resistance to the development of IDDM. The first susceptibility axis (S1) is primarily associated with DW3-DR3 and the second susceptibility axis (S2) with DW4-DR4. All the other factors of the A, C, B, C<sub>4</sub>, C<sub>2</sub> and Bf loci are secondarily increased because of linkage disequilibrium within the HLA complex. The axis of resistance (R) is primarily associated with DW2-DR2 with secondary increases in B7 and A3.

All the above associations are only valid for Caucasian populations.

\*B15 is now subdivided into BW62 and BW63

\*B40 is now subdivided into BW60 and BW61

Modified from Cudworth and Wolf (1982)

'association' theory implies that HLA alleles are linked to one or more alleles responsible for the predisposition to develop IDDM: for example, that immune response genes might exist within the HLA system and be in linkage disequilibrium with the genes of the DR locus (McDevitt and Bodmer, 1974). The existence of such immune response (Ir) genes is established in the mouse, in which animal they are linked to the genes for the major murine histocompatibility system (the H-2 complex) (Moller, 1978); by analogy it can be postulated that a similar condition exists in man, and that Ir genes associated with the DR locus alleles confer a genetically controlled immune response which leads to the state of IDDM in susceptible individuals or protection from IDDM in resistant individuals. The modern evidence which has formulated the concept of genetic heterogeneity within IDDM would favour the existence of two separate Ir genes in strong linkage disequilibrium with the S1 and S2 HLA axes, probably with the DR3 and DR4 alleles (figure 7/2) and a third Ir gene or genes, in linkage disequilibrium with DR2, conferring protection against development of IDDM. As yet, there is no way to identify immune response genes in man, so the hypothesis must still be sub judice. However, it is intriguing to speculate that HLA antigens act as markers for immune response genes in analogous fashion to the presence of islet-cell antibodies as markers for the development of IDDM. Thus the possession of an HLA susceptibility axis confers a high risk of IDDM; the appearance of ICA in an individual implies that the process of immunologically-mediated beta cell destruction has already started and that IDDM may ultimately ensue. This is certainly the kind of inference that can be drawn from the various/

various studies reviewed in chapter two, where the presence of both 'markers' was demonstrated in subjects who developed IDDM over varying (sometimes lengthy) periods of time. The mode of action of these postulated immune response genes remains speculative. Possibly they control the immune response to environmental factors capable of initiating damage to the pancreatic islet beta cell membrane, e.g. viruses, but evidence for this will not be obtained until the Ir genes in man are identified and characterised.

Is there heterogeneity of immune response within IDDM? The concept of two separate HLA-DR associated susceptibility genes might lead one to expect that there may be separate clinical associations between DR3 and DR4 with immunological or clinical aspects of the disease. The strong clinical associations between insulin-dependent diabetes and organ-specific autoimmunity have already been reviewed in chapter one, and it will be recalled from chapter two that HLA-B8 has a significant positive association with idiopathic Addison's disease, Grave's disease, primary hypothyroidism and Hashimoto thyroiditis. It is difficult to believe that the association of HLA-B8 with so many different autoimmune diseases is coincidental. The evidence which indicates that HLA-B8 is associated with persistence of islet-cell antibodies in insulin-dependent diabetics has been discussed earlier (chapter two); many of these patients examined by Irvine et al (1977a) and Cudworth et al (1980) had coexistent overt autoimmune disease, while both these studies identified a similar group of B8-positive diabetics with polyendocrine antibodies (thyroid, gastric, adrenal) rather than overt disease. The clear inference to be drawn from all these associations is that gene(s) in the HLA chromosomal/

chromosomal region in linkage disequilibrium with HLA-B8 may confer peculiar susceptibility to organ-specific autoimmunity, including an autoimmune form of diabetes. In other words, the HLA susceptibility axis for insulin-dependent diabetes which includes HLA-B8 is the axis which permits the development of autoimmune diabetes. Logically, one would expect an equally striking association between HLA-DR3 and autoimmunity to be found in these diabetics; as noted in chapter two, studies of combined HLA-B and -DR typing in diabetics are sparse but one recent and striking observation was the finding by Gudworth and Wolf (1982) that seventeen of eighteen patients with coexistent insulin-dependent diabetes and Grave's disease were HLA-DR3 and -B8 positive. Naturally there was a striking female preponderance in these patients, many of whom have persistent islet-cell or other organ-specific autoantibodies. By analogy with other autoimmune diseases, it can be anticipated that a patient population of true autoimmune diabetics should contain an excess of females.

By contrast, alleles of the second HLA axis of susceptibility to insulin-dependent diabetes do not seem to confer any strong association with autoimmunity. HLA-B15 (now subdivided into BW62 and BW63) which is in linkage disequilibrium with DR4, is associated with different immunological responses in diabetes, including increased titres of antibodies to exogenous insulin injections (Bertrams et al, 1976; Ludwigsson et al, 1977; Schernthaner et al, 1977; Irvine et al, 1978b) and perhaps with increased titres of antibodies to Cocksackie B4 virus (Gudworth et al, 1977). Much work needs to be done to further define the concept of immune heterogeneity in IDDM, and to examine non-Caucasian/



non-Caucasian populations of IDDM (in which B8 and BW62/BW63 are rare) for relationships between HLA and autoimmune or other immune phenomena. Some preliminary studies of this nature have been undertaken in Japan where the prevalence of IDDM is much lower than the West (West, 1978). HLA-DW3, -DR3 and -DR4 are associated with IDDM in Japanese, but HLA-B8 is virtually absent in this ethnic group. It was found that IDDM Japanese patients had a much lower prevalence of cytoplasmic ICA (14% within one year following diagnosis) than their equivalents in a Caucasian population, and that ICA did not persist beyond four years following diagnosis (Irvine, 1980). Clearly it will be important to extend these observations to other ethnic groups in which HLA-B8 is rare, and to see whether a presumably weak autoimmune component is associated with a lower prevalence of IDDM in such populations.

#### 7/4 Summary and conclusions

In conclusion, it is appropriate to recall the criteria originally proposed for 'autoimmune diabetes mellitus' in the Introduction to this thesis (table 1/3); and it is now possible to state that an impressive weight of direct and indirect evidence suggests that autoimmune mechanisms have an important role in the aetiology of some cases of insulin-dependent diabetes mellitus in man.

With regard to the indirect evidence, insulin-dependent diabetes is commonly associated with overt or subclinical autoimmune disease and may appear as part of a polyendocrine autoimmune syndrome. Organ-specific autoantibodies are frequently found in sera from insulin-dependent diabetics, directed especially against antigens in the thyroid or stomach, and are present to excess in the relatives of these patients/

patients. The high prevalence of thyrogastric antibodies in insulin-dependent diabetes is independent of the age of onset of clinical disease, duration of treatment or the presence of specific diabetic complications. All these facts suggest that autoantibody production is an integral part of the diathesis of insulin-dependent diabetes. Insulin-dependent diabetics often possess the same HLA factors that are found in patients with thyroid or adrenal autoimmune disease, especially HLA-B8, and these HLA factors are probably linked with immune response genes which permit the full expression of an autoimmune response.

With regard to the direct evidence, insulinitis seems to be a relatively common finding in the pancreas of newly diagnosed insulin-dependent diabetics and has inevitably been compared with the very similar round-cell infiltrates found in organ-specific autoimmune disease affecting the adrenal, thyroid and stomach. The autoimmune analogy is further strengthened by the findings in human diabetes of cell-mediated immunity to porcine, bovine and human pancreatic antigens (chapters three and four) and islet-cell autoantibodies (chapter two). The presence of islet-cell antibodies has been documented in individuals with normal or mildly-impaired carbohydrate tolerance who have subsequently progressed to develop typical ketosis-prone insulin-dependent diabetes. All these studies lend strong credibility to the concept of a true autoimmune form of diabetes mellitus, which has now been shown to fulfil the classical criteria for the disease (close relationship with other autoimmune diseases, lymphocytic infiltration of the end-organ, circulating humoral antibody directed against the end-organ, circulating lymphocytes sensitised against the end-organ). For an external or environmental factor to initiate the autoimmune process in/

in susceptible individuals, one may turn to the epidemiological studies suggesting an association between insulin-dependent diabetes and viral infections, especially mumps and Coxsackie, and the pathological evidence which has demonstrated Coxsackie virus as a cause of insulinitis or even of insulin-dependent diabetes in a few cases.

At the end of 1974, shortly after the first descriptions of pancreatic cytoplasmic islet-cell antibody, it was possible for the author to review the concept of autoimmune diabetes mellitus in The Lancet and to summarise thus: 'it may not be too fanciful to propose a unifying hypothesis whereby young subjects, who have inherited a particular complex of immune-response genes by virtue of their HLA type, respond to (say) a viral infection by initiating an irreversible and relentless series of autoimmune processes which culminate in destruction of the endocrine pancreas and the development of clinical diabetes mellitus' (MacGuish, 1974). With hindsight, it can be said that the basic hypothesis may be valid but is an oversimplification. We now appreciate that insulin-dependent diabetes differs from the classical autoimmune diseases in several important respects. First, there is no recognised association between any common viral infection and the subsequent development of classical or organ-specific autoimmune disease. Second, the age distribution of IDDM is distinctly different from the classical autoimmune diseases, the majority of cases occurring in young persons and the minority in the elderly. Third, islet-cell antibody is never found in some patients (say 15 to 20%), is a transient phenomenon in many others and persists in only a minority of patients (with coexistent autoimmune disease). Fourth, cell-mediated antipancreatic autoimmunity, albeit/



albeit in an assay system using unseparated leucocytes and tumour-derived antigen, can only be detected in about 50% of patients with IDDM at or shortly after the diagnosis of clinical disease. Fifth, there is an association of IDDM with HLA-DW4-DR4 which is not found with the classical autoimmune diseases. All these facts clearly imply that genetic and immunological heterogeneity is a feature of insulin-dependent diabetes and that autoimmunity may be the dominant factor in only one subgroup of patients.

Appreciation of these factors has led to a difficulty in incorporating all this new knowledge of aetiological factors within a framework which not only allows a better classification of insulin-dependent diabetes but also takes account of the need for any classification to be both comprehensive and flexible with regard to the relative importance of HLA, autoimmunity, viruses, etc. in individual cases. The earlier classification of the WHO Expert Committee on diabetes mellitus (1965), which subdivided patients by age of onset into 'juvenile-onset diabetes' (insulin-dependent) and 'maturity-onset diabetes' (insulin-independent) was clearly inappropriate and has been subjected to rigorous criticism by workers who were intimately involved with many of the studies referred to in this thesis. Cudworth (1976) first proposed removal of classification by age and called juvenile-onset or insulin-dependent diabetes 'Type 1' to distinguish it from 'Type 2' or maturity-onset (insulin-independent) diabetes. Bottazzo and Doniach (1976) used this numbering system to classify subtypes of insulin-dependent diabetes as 'Type 1a' (possibly due to viral infection) and 'Type 1b' (possibly due to autoimmunity).

The/



The same workers subsequently refined and amplified the features of this classification as further information was forthcoming from HLA and immunological studies (Cudworth et al, 1980; Cudworth and Wolf, 1982), but retained the same numerical system. Irvine (1977) proposed a subdivision of Type 1 diabetes into three categories according to whether the main factor was a genetically determined diathesis towards islet-cell autoimmunity (Type 1a), towards islet cell damage by appropriate viral infection or other agent in the absence of islet-cell autoimmunity (Type 1c), or a combination of the two diatheses (Type 1b). Irvine's classification also took into account the knowledge that ICA-positive diabetics could pass through a clinical phase of milder diabetes, controlled by oral hypoglycaemic drugs, but subsequently progressing to insulin-dependent diabetes, by classing such patients as Type 1 diabetics. The views of all these authorities were later considered at an international workshop set up by the National Diabetes Data Group (NDDG) of the National Institutes of Health, U.S.A., to develop a nomenclature and classification system for diabetes mellitus (NDDG, 1979). In essence, the NDDG favoured the grouping of all insulin-dependent patients into Type 1 diabetics (IDDM), without further numerical subdivision, but recognised the association of environmental and HLA factors and abnormal immune responses, including autoimmune reactions with this type of diabetes. A simplified version of this classification scheme was subsequently adopted by the second report of the WHO Expert Committee on Diabetes Mellitus (1980) and is now coming into general use (table 1/4). Further analysis of the merits of the various classifications proposed for Type 1 diabetes is beyond the scope of this thesis but the author would submit that it is/

is important for any classification to recognise that the different aetiologic factors in Type 1 diabetes (genetic susceptibility, viral infections, immunological response, etc.) are likely to exert a spectrum of influence in individual cases rather than being confined to discrete subtypes. Thus in some cases a major autoimmune and minor environmental influence may interact, in others the environmental factor (say severe viral infection) may predominate, and so forth. Moreover, the influence of these factors which initiate beta cell damage may long predate the onset of clinical diabetes, far less the need for exogenous insulin injections. All these considerations will always make it difficult to judge the relative importance of different aetiologic factors in the genesis of Type 1 diabetes but it seems safe to suggest that autoimmune responses alone, or viral infection alone, will only induce diabetes in a minority of patients.

Finally, any practical applications of the studies described and reviewed in this thesis remain to be discussed. It appears to the author that these fall into two categories. For the present, the demonstration that poorly-controlled diabetes impairs immunologic function should encourage clinicians to achieve the best possible metabolic control for patients under their care; this can be aided by modern methods of home blood glucose monitoring and measurements of glycosylated proteins in diabetes. Second, the demonstration that islet-cell antibody is a marker for eventual insulin-dependent diabetes is an important observation that would have immediate clinical application if a standardised, reproducible antibody assay was developed for general hospital laboratory usage. For the future, the recognition that a relatively slow immune-mediated destruction of the beta cell/

ell is taking place before the onset of insulin-dependent diabetes,  
r that viral infection may long predate the disease, offers oppor-  
unity for immune intervention at the time before beta cell damage is  
irreversible. In the present state of knowledge, current immuno-  
suppressive techniques could not be applied with safety to such  
individuals, but further characterisation of the pancreatic islet-  
cell antigen-antibody system might permit a rational form of immuno-  
therapy to be devised and exhibited before irreversible damage is done  
to the pancreas. The record of the past gives every reason to hope  
that the next ten years will be marked by major advances in our under-  
standing of the genesis of insulin-dependent diabetes mellitus and  
ipso facto the appropriate means of its prevention.

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## APPENDIX I

### LABORATORY METHODS AND TECHNIQUES

(Where not fully described in the relevant chapters)

#### I/1 Buffers and reagents, etc.

##### I/1a Sterile phosphate buffered saline

To one litre of distilled water, add the following chemicals (all obtained from British Drug Houses Ltd., 'Analar' grade):

Potassium dihydrogen orthophosphate - 0.34 g

Dipotassium hydrogen orthophosphate - 1.21 g

Sodium chloride - 8.0 g

Sterilise the solution by autoclaving at 115°C for ten mins.

##### I/1b Ficoll/Triosil preparation

Used for the separation of human lymphocytes from whole blood by density centrifugation.

#### Materials

Ficoll powder (obtained from Pharmacia AB or Sigma Ltd).

Triosil '75' - 75% solution (obtained from Glaxo Ltd).

#### Method

Dissolve 9.557 g Ficoll in 80 ml distilled water.

Add 20 ml (1 ampoule) Triosil.

Add water until specific gravity of solution is 1.076-1.078 at room temperature.

Sterilise by autoclaving at 115°C for ten minutes.

Store in dark, in coldroom or refrigerator. Do not freeze.

Bring to room temperature before use.

##### I/1c Eagle's/

## I/1c Eagle's Basal Medium (EBM)

Used for the in vitro cultivation of human cells, with serum added as a growth-promoting medium. EBM has the following composition at working strength:

<u>Amino acids</u>	mg/litre	<u>Vitamins</u>	mg/litre
l-arginine	21.0	aneurine	1.0
l-cystine	12.0	choline	1.0
l-histidine	9.5	folic acid	1.0
dl-isoleucine	52.0	inositol	2.0
dl-leucine	52.0	nicotinamide	1.0
dl-phenylalanine	33.0	pantothenate	1.0
dl-threonine	48.0	pyridoxal	1.0
dl-tryptophane	8.0	d-biotin	1.0
l-tyrosine	18.0	riboflavin	0.1
dl-valine	47.0		
<u>Inorganic salts</u>	mg/litre	<u>Miscellaneous</u>	mg/litre
sodium chloride	6800	glucose	1000
potassium chloride	400	l-glutamine	292
calcium chloride	200	phenol red	10
magnesium sulphate	200	NaHCO <sub>3</sub>	1100
sodium phosphate	150		

EBM is conveniently obtained from Wellcome Reagents Ltd. (Catalogue TC30) as a sterile working solution with added antibiotics (penicillin  $2 \times 10^5$  units/litre, streptomycin  $1 \times 10^5$  µg/litre).

## I/2 Indirect immunofluorescence technique for antibody detection.

This technique, which was first introduced by Coons et al, employs/

employs an antibody labelled with a fluorescent dye to locate the complementary antigen in microscopic preparations. The site of the antigen:antibody interaction is localised by fluorescence microscopy (e.g. thesis, figure 5/1). In this way fluorescein-labelled class specific antibodies to human IgG, IgM and IgA are used to demonstrate the presence and define the class of human immunoglobulin synthesised within cells or fixed on to cell surfaces.

In the studies described in this thesis, the indirect immunofluorescence technique has been used to identify circulating auto-antibodies against thyroid cell cytoplasm, gastric parietal-cell cytoplasm, adrenal cortex, pancreatic islet-cell, cell nuclei (ANF) and mitochondria.

#### Method

1. Snap-frozen blocks of the required tissue substrate (thyroid, kidney, etc.) are cut into thin (approx. 5 micron) sections in a cryostat at  $-20^{\circ}\text{C}$ . The sections are transferred onto clean glass slides (76 x 25 mm, thickness 0.8-1.0 mm) and allowed to air-dry at room temperature.
2. The sera for testing are removed from storage in a deep-freeze and thawed in a water bath at a temperature of  $34-37^{\circ}\text{C}$ . This temperature range allows rapid thawing and does least damage to the immunoglobulins.
3. The prepared slides are placed in Shandon trays, moistened with a small amount of veronal buffer.
4. Each test serum sample is mixed by gently inverting two or three times. A generous aliquot of each sample is applied to the appropriate slide using a platinum loop. The loop is flamed to sterilise between each/

each serum sample.

5. When all the test sera have been applied to the appropriate sections, the slides are allowed to incubate for thirty minutes.

6. At the end of this time, the sections are thoroughly washed by dipping the slides in veronal buffer (pH 7.2) in a Coplin jar; thereafter the slides are placed in a washing bath containing the same buffer and washed for twenty minutes.

7. The slides are then transferred to a fresh bath of veronal buffer for a further fifteen-minute wash.

8. Each slide is then removed separately from the washing bath and dried, leaving only the section in a pool of veronal buffer.

9. Using the platinum loop, one drop of the required antihuman Ig-fluorescein conjugate is applied to each slide, in sufficient quantity to cover the entire section. The fluorescent-labelled antibodies are all obtained from Wellcome Reagents Ltd., and the following classes are available: anti-human IgG (Reference MF 03); anti-human IgM (Reference MF 04); anti-human IgA (Reference MF 05). A polyvalent conjugate (anti-human IgG, IgM, IgA) is also available (Reference MF 01). Many circulating autoantibodies are of IgG class (e.g. thyroid/gastric microsomal, pancreatic islet-cell).

10. The sections are allowed to incubate with the labelled antibody, in Shandon trays, for twenty minutes.

11. At the end of this time, the sections are washed as before by dipping the slides in a Coplin jar of veronal buffer before being washed for twenty minutes in the same buffer.

12. The slides are then transferred to a fresh bath of veronal buffer for a further fifteen-minute wash.



13. The slides are finally removed from the washing bath, dried as before (i.e. without damaging the section), and the sections are mounted using 10% glycerol and 22 x 22 mm Chance No 0 coverslips.

14. The prepared slides are left to air-dry. Thereafter they are examined with a u.v. microscope for evidence of fluorescence.

### I/3 Tanned cell haemagglutination test for thyroglobulin antibody

This test is used for the quantitative measurement of auto-antibodies to human thyroglobulin. Thyroglobulin is extracted from human thyroid by classical salt precipitation techniques and coupled to sheep red cells: if these coated cells are incubated in the presence of antibody-containing serum they are agglutinated, yielding an even carpet of cells at the bottom of a tube or perspex cup. Lack of agglutination (i.e. with antibody-negative serum) is indicated by the cells settling into a tight button.

Longterm stability of the tanned coated cells used in the test is achieved by formalisation of the final suspension. As a proportion of human sera contain heterophile antibodies capable of agglutinating sheep cells, control tanned sera (uncoated with thyroglobulin) are also used to test each serum.

#### Reagents

1. Thyroglobulin Haemagglutination Test Kit (Wellcome Reagents Ltd., Catalogue No. AD 02). Contains 35 ml 1% suspension of formalised, tanned sheep red blood cells coated with human thyroglobulin in a borate buffer, and an identical quantity of a suspension of uncoated tanned red cells.

2. Normal saline (0.85% solution).

3/

3. A positive control human serum (containing thyroglobulin antibody in a titre of at least 1:2500) and a negative control human serum.

#### Apparatus

1. Test tubes (3" x  $\frac{1}{2}$ " pyrex) and metal test-tube rack.
2. Oxford or Eppendorf 100 microlitre pipette with disposable plastic tips.
3. Graduated pipette (10 ml) for addition of saline to wells, and 1 ml graduated pipette.
4. Agglutination trays (WHO perspex plates).

#### Method

1. Pipette 0.1 ml of each serum sample (including the positive control of known titre) into a pyrex test tube.
2. To each test tube add 0.4 ml control cells from the Wellcome kit.
3. Mix well, allow to stand on the bench for 15 minutes, then centrifuge for 10 minutes at 2000 rpm. The supernatant after centrifugation (i.e. a 1:5 dilution of serum free from sheep cell agglutinins) is used for the test.
4. Into each row of the WHO plate (which contains 8 rows of wells, 10 wells per row) pipette 0.1 ml volumes of the following serum dilutions:  
 $1/5$ ;  $1/25$ ;  $1/250$ ;  $1/25000$ ;  $1/250000$ ;  $1/2500000$ ; and  $1/5$  (for the control well).
5. Add 0.1 ml of thyroglobulin sensitised (coated) cells to each of the first seven wells and 0.1 ml control (uncoated) cells to cup eight.
6. Mix cells and sera by very gentle shaking, cover with a glass plate/

plate and allow to stand undisturbed at room temperature for at least six hours (preferably overnight) before reading.

### Results

The thyroglobulin titre in a test serum is the highest dilution at which all the cells form a diffuse carpet over the bottom of the well. A negative test appears as a small circle, or compact button of cells, at all dilutions.

The control well (well eight) must always give a clear-cut negative result. If this well shows agglutination, the test serum must be reabsorbed and then retested.

The Lancet, Saturday 30 December 1974

APPENDIX II

**PUBLISHED PAPERS SUBMITTED IN SUPPORT OF THESIS**



# ANTIBODIES TO PANCREATIC ISLET CELLS IN INSULIN-DEPENDENT DIABETICS WITH COEXISTENT AUTOIMMUNE DISEASE

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**Summary** Using an indirect immunofluorescence technique, circulating antibodies to pancreatic islet cells were found in the sera of 5 patients with insulin-dependent diabetes mellitus and coexistent autoimmunity. All these patients had at least one major overt autoimmune disease, and all had a variety of organ-specific antibodies in the serum. In each case the islet-cell antibody was of IgG class. These findings provide further direct evidence to support the hypothesis of an autoimmune form of diabetes mellitus.

## Introduction

CONSIDERABLE interest has lately been expressed in the possibility that autoimmunity may have some aetiological role in a proportion of cases of juvenile-onset diabetes mellitus. Indirect evidence to support such a hypothesis has been provided by observation of the clinical association between diabetes and other diseases of established or putative autoimmune aetiology (pernicious anaemia,<sup>1,2</sup> thyrotoxicosis,<sup>3,4</sup> Hashimoto thyroiditis,<sup>5,6</sup> primary hypothyroidism,<sup>7,8</sup> idiopathic Addison's disease,<sup>7,9</sup> idiopathic hypoparathyroidism,<sup>9,10</sup> and myasthenia gravis<sup>11</sup>). Similarly, epidemiological studies have confirmed an excess prevalence of humoral organ-specific autoantibodies in diabetics when compared with age and sex matched non-diabetic populations—e.g., circulating antibodies to thyroid and gastric parietal cytoplasm are found two to four times more frequently in diabetics than non-diabetics,<sup>2,12,13</sup> the prevalence of antibodies to intrinsic factor is increased at least fourfold in diabetics over matched control populations,<sup>1,2</sup> and the prevalence of adrenal antibodies, which are rare in the absence of idiopathic Addison's disease, has been estimated to be over thirty times higher in diabetic than non-diabetic populations.<sup>14</sup> In all cases the excess antibody prevalence is mainly associated with female sex, insulin dependency, and juvenile-onset diabetes. More direct evidence in support of an autoimmune hypothesis has been obtained from in-vitro studies of

cellular immunity in diabetes; antipancreatic cellular hypersensitivity has been demonstrated by the leucocyte-migration technique in approximately 30% of diabetics—mainly recently diagnosed insulin-dependent patients<sup>15-17</sup>—and blastogenic transformation of lymphocytes from untreated or newly-diagnosed diabetics has been induced by prolonged culture of these cells in the presence of insulin or insulin-B-chain antigens.<sup>18</sup> These and other in-vitro studies indicate the existence in diabetics of lymphocyte subpopulations, presumably T lymphocytes, sensitised against antigens of the endocrine pancreas or its secretions. Direct evidence comes from the pathological finding of "insulitis" (lymphocytic infiltration of the islets of Langerhans) in the pancreas in untreated young diabetics who died shortly after the onset of their disease,<sup>19</sup> although this lesion is not accompanied by the plasma-cell infiltration and lymphoid-follicle formation that is regarded as typical of the accepted organ-specific autoimmune diseases.<sup>20</sup>

One major difficulty in applying the autoimmune hypothesis to diabetes mellitus has been the failure to find humoral autoantibodies, directed against the endocrine pancreas, in sera of diabetics. Early investigators, using haemagglutination, gel diffusion, and precipitation techniques,<sup>21,22</sup> claimed to find antipancreatic antibodies in the sera of many of the diabetics they tested, as well as in other forms of chronic pancreatic disease. However, these results were not confirmed,<sup>23</sup> and were probably artefacts. We have previously searched with an immunofluorescent technique for such an antibody in sera from several hundred diabetics, of all clinical types, without success,<sup>2,24</sup> and others<sup>25,26</sup> have had a similar experience. However, we can now describe the detection of circulating antibodies against the islets of Langerhans in sera from a number of patients in whom insulin-dependent diabetes coexisted with idiopathic Addison's disease and other autoimmune disorders.

## Patients and Methods

### Patients

Sera from the following groups of patients and controls were tested for islet-cell antibodies:

- (A) 20 juvenile-onset insulin-dependent diabetics with co-existent Addison's disease. All had circulating adrenal antibodies and all but 2 were women. Apart from adrenal failure, 9 had one or more additional autoimmune disease (thyrotoxicosis, Hashimoto thyroiditis, primary hypothyroidism, pernicious anaemia, and premature ovarian failure), accompanied by the appropriate circulating antibodies.
- (B) 20 juvenile-onset insulin-dependent diabetics with cir-

culating thyroid and/or gastric parietal cytoplasmic antibodies, but no evidence of overt autoimmune disease. These patients were matched as closely as possible for age, sex, and duration of diabetes with the patients in group A.

(C) 25 juvenile-onset insulin-dependent diabetics with no organ-specific antibodies in the serum.

(D) 40 non-diabetic controls with no organ-specific antibodies in the serum.

#### Detection of Islet-cell Antibodies

Fresh human pancreas was used as the tissue substrate for these experiments. The tissue was snap frozen immediately after removal, stored in small blocks at  $-40^{\circ}\text{C}$ , and cut as required into thin ( $5-7\text{ }\mu\text{m}$ ) sections on a cryostat. All tests were performed on air-dried, unfixed sections. Islet-cell antibodies were detected by the indirect immunofluorescence technique,<sup>27</sup> using antihuman IgG fluorescein-isothiocyanate (F.T.C.) conjugate (Wellcome) at a 1/14 dilution. All sera were initially tested undiluted, but those giving positive results were subsequently retested in serial dilutions to establish the antibody titres. Positive sera were further tested with anti-IgA and anti-IgM conjugates to ascertain the immunoglobulin class of the antibodies. Experiments were also undertaken to confirm that the fluorescence observed with anti-IgG conjugate could be abolished by pretreatment of the pancreatic sections with unlabelled anti-human IgG.

#### Other Autoantibody Studies

Using techniques previously described,<sup>2</sup> all sera were tested for antibodies to thyroglobulin, thyroid cytoplasm, gastric parietal cytoplasm, cell nuclei, and mitochondria. In addition sera from patients in group A with premature ovarian failure were examined<sup>28</sup> for antibodies reactive with extra-adrenal steroid-producing cells (ovary, testis, and placenta), and those with pernicious anaemia were examined for intrinsic-factor antibodies.<sup>2</sup>

### Results

#### Islet-cell Antibodies

Cytoplasmic immunofluorescence of the pancreatic islets was detected in 5 of the 105 sera tested, all from patients in group A (table 1). In each case the fluorescence seemed to involve the islet cells diffusely, being present in  $\alpha$  and  $\delta$  cells in addition to  $\beta$  cells. Immunofluorescence was detected using undiluted serum only in 1 case, the antibody titre ranging from

TABLE 1—ISLET-CELL ANTIBODIES DETECTED BY IMMUNOFLOUORESCENCE IN DIABETIC PATIENTS

Group	Patients tested	Islet-cell antibody	
		Positive	Negative
A	Diabetic with Addison's disease ..	5	15
B	Diabetic with thyrogastric antibodies ..	0	20
C	Diabetic with negative autoantibodies ..	0	25
D	Non-diabetic with negative autoantibodies ..	0	40
	Total ..	5	100

1/4 to 1/128 in the other 4 sera (table II). Some variation in the intensity of fluorescence within individual islet cells was observed with the two strongest sera, but in all cases the islets stood out brightly from surrounding exocrine tissue. In each serum the antibody was shown to be of IgG class, immunofluorescence being negative with anti-IgA and anti-IgM conjugates, and it was also possible to abolish the fluorescence by pretreatment of the pancreatic sections with unlabelled anti-IgG. With negative sera the pattern of fluorescence was entirely different; the islets either merged with exocrine tissue or were only distinguished with difficulty, and any fluorescence observed was either an orange-pink autofluorescence or occasional faint cytoplasmic fluorescence, sporadically distributed in both endocrine and exocrine tissue.

#### Other Autoimmune Lesions in Patients with Islet-cell Antibodies

The clinical and serological findings in the 5 cases with islet-cell antibodies are presented in table II. As stated, all were diabetics with idiopathic Addison's disease and 4 had additional overt autoimmune disease or diseases. The 3 patients with premature ovarian failure had a multiplicity of steroid-cell antibodies reacting with different antigens in ovary, placenta, and testis, typical of the serological findings in this polyendocrine syndrome.<sup>29</sup>

### Discussion

We have detected an antibody to pancreatic islet

TABLE II—CLINICAL AND SEROLOGICAL FINDINGS IN DIABETICS WITH ISLET-CELL ANTIBODIES

Age	Sex	Clinical conditions	Age of onset (yr.)	Autoantibodies										
				Islets (titre)	Adrenal (titre)	Thg (titre)	T.C.	G.P.C.	I.F. (titre)	A.N.A.	Mito	Ovary	Placenta	Testis
44	F	Thyrototoxicosis, diabetes mellitus, Addison's disease, amenorrhoea	22 30 36 36	128	4	—	—	+	—	—	—	+	+	+
43	F	Amenorrhoea, pernicious anaemia, primary hypothyroidism, Addison's disease, diabetes mellitus	28 33 40 40 43	64	8	250	+	+	94 u/ml.	—	—	+	+	+
33	F	Hashimoto thyroiditis, Addison's disease, diabetes mellitus	29 29 31	8	16	25,000	+	+	—	—	—	N.T.	N.T.	N.T.
32	F	Diabetes mellitus, Addison's disease	26 31	4	8	—	—	—	—	—	—	N.T.	N.T.	N.T.
45	F	Oligomenorrhoea, primary hypothyroidism, diabetes mellitus, Addison's disease	30 31 44 45	Undiluted serum only	+	—	+	—	—	—	—	+	+	—

Thg = antibodies to thyroglobulin. T.C. = antibodies to thyroid cell. G.P.C. = antibodies to gastric parietal cell. I.F. = antibodies to intrinsic factor. A.N.A. = antibodies to cell nuclei. Mito = antibodies to mitochondria. + = antibody present, titre not determined. — = antibody not present. N.T. = serum not tested for antibody.



cell by immunofluorescence in 5 out of 20 insulin-dependent diabetics with coexistent Addison's disease whose sera we have studied so far. Earlier attempts, including our own,<sup>2,24-26</sup> may have failed because sera from diabetics with overt autoimmune disease of other organs were deliberately excluded while sera from patients with maturity-onset diabetes, in which an autoimmune aetiology is unlikely, were included. Our findings suggest that islet-cell antibody cannot be found in diabetics whose serum contains no other antibodies or even in those who merely possess circulating thyrogastric or other antibodies, but is confined to patients with one or more major overt autoimmune diseases, in addition to diabetes mellitus.

We have not yet examined sera from insulin-independent diabetics with autoimmune disease for the presence of islet-cell antibody, and it is important to consider whether the presence of such antibodies is related to previous insulin therapy. This seems most unlikely since the antibody was not detected in insulin-dependent patients who did not have overt autoimmune disease, and the pattern of islet-cell fluorescence with positive sera was completely different from that previously reported<sup>29</sup> (but not subsequently confirmed) for insulin antibodies.

Further studies are needed to confirm the organ-specificity of islet-cell antibody and to characterise the nature and location of the antigen(s) against which it is directed. It is too early to speculate on a role for such antibodies in the aetiology of diabetes, although it is tempting to postulate that their presence may be associated with the insulinitis seen in some cases of juvenile-onset diabetes. An accurate estimate of the frequency of islet-cell antibody will not be possible until larger numbers of sera from diabetics with autoimmune disease, and indeed from non-diabetics with autoimmune disease, have been tested. However, it seems reasonable to conclude that islet-cell antibodies are uncommon in diabetics, that they probably occur only in patients with overt autoimmunity, but that their existence may provide further direct evidence to strengthen the concept of "autoimmune diabetes mellitus".

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Requests for reprints should be addressed to A. C. M.

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#### Addendum

G. F. Bottazzo, A. Florin-Christensen, and D. Doniach (*Lancet*, 1974, ii, 1279) have lately reported the detection of antibodies to pancreatic islet cells in the sera of 13 patients with multiendocrine deficiencies associated with organ-specific immunity. 10 were diabetic.

# THE LANCET

## Autoimmune Diabetes Mellitus

HORMONE-PRODUCING tissue seems peculiarly vulnerable to assault by autoimmune disease mechanisms, and it is understandable that an aetiological role for autoimmunity should be sought in an endocrinopathy as common as diabetes mellitus. The evidence suggesting an autoimmune basis for certain types of diabetes has been marshalled from several sources. Firstly, there are the clinical observations that diabetes coexists with overt autoimmunity more often than can be accounted for by chance. Thyrogastric autoimmune diseases such as pernicious anaemia,<sup>1,2</sup> thyrotoxicosis,<sup>3</sup> Hashimoto thyroiditis,<sup>4</sup> and primary hypothyroidism<sup>5,6</sup> are all said to be encountered with increased frequency in diabetics and indeed in their first-degree relatives.<sup>2</sup> The most impressive example is idiopathic Addison's disease (autoimmune adrenalitis), in which the prevalence of overt diabetes is around 14%<sup>7</sup>—i.e., about six times higher than in background population. Diabetes may coexist with multiple autoimmune disorders, notably the combination of adrenalitis and thyroiditis (Schmidt's syndrome<sup>5</sup>), and may appear in the rare familial endocrinopathies.<sup>8</sup>

These clinical observations are complemented and largely confirmed by work on circulating organ-specific humoral autoantibodies. Most investigators report a twofold to fourfold increase in the prevalence of thyrogastric cytoplasmic antibodies in diabetic patients,<sup>1,2,9</sup> intrinsic-factor antibody occurs with similar

frequency,<sup>1,2</sup> and adrenal antibody may be thirty times more common in diabetic than in nondiabetic populations.<sup>7</sup> As with overt autoimmune diseases, excess antibody prevalence is particularly associated with insulin-dependent juvenile-onset diabetes, predominantly in women, and sera from the first-degree relatives of these diabetics show a similar antibody excess.<sup>10</sup> Further genetic evidence to link diabetes with autoimmunity has been derived from studies of histocompatibility-antigen (HL-A) typing: HL-A8 is so frequent in juvenile-onset insulin-taking diabetics that it has been proposed as a genetic marker for the disease,<sup>11</sup> and the same antigen has been detected with similar frequency in thyrotoxicosis<sup>12</sup> and idiopathic Addison's disease.<sup>13</sup> Complexes like HL-A carry not only the genes controlling serologically detectable antigens but also immune-response (Ir) genes which control the development of cell-mediated immunity to certain antigens, and it is intriguing to speculate that the Ir genes associated with HL-A8 are those which permit the full expression of an autoimmune response, irrespective of the stimulus which elicits this response.

Direct evidence for the autoimmune hypothesis comes mainly from work on pancreatic abnormalities and on cell-mediated immunity in diabetes. Lymphocytic infiltration of the islets of Langerhans in the pancreases of young diabetics was first recognised by SCHMIDT in 1902,<sup>14</sup> but this lesion, termed insulinitis by VON MEYENBURG,<sup>15</sup> was thought to be extremely rare until it was detected by GEPTS<sup>16</sup> in 15 of 22 patients who died of acute diabetes. The pancreases examined by GEPTS were all removed from patients aged under thirty who had died less than six months after the onset of diabetic symptoms and who were mostly untreated before death. Insulinitis in this selected group has come to be regarded as a common lesion, although its exact frequency is a matter of conjecture<sup>17</sup> and the advent of insulin treatment makes it unlikely that any modern pathologist will now see enough material to arrive at an accurate figure. What does seem certain is that insulinitis is a transient lesion that has never been found in juvenile diabetics who died more than a year after the onset of symptoms.<sup>16,17</sup> The temptation to compare insulinitis with the end-organ changes (adrenalitis, thyroiditis) in accepted autoimmune disease is strong, and the belief that this lesion may also have an immune basis is supported by experi-

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mental data: RENOLD et al.<sup>18</sup> induced a true autoimmune insulinitis by injecting cattle with homologous insulin, and a particularly striking model of experimental autoimmune diabetes has lately been described by NERUP et al.<sup>19</sup> who found that injection of homologous endocrine pancreas suspensions in rodents was followed by the appearance of insulinitis, antipancreatic cellular hypersensitivity, and carbohydrate intolerance. There are, however, equally powerful epidemiological<sup>20</sup> and experimental<sup>21</sup> grounds for believing that insulinitis in human diabetes may be due to viral infection of the endocrine pancreas, possibly with group-B Coxsackie or other common viruses; conceivably the real solution to the question of what causes insulinitis is that the immunological and viral hypotheses are not mutually exclusive, and that virus infection in man can serve as the mediator to trigger a series of immunological reactions which result in insulinitis.

Investigations of lymphocyte function provide the fourth support for the hypothesis of autoimmune diabetes. The absolute numbers of circulating T and B lymphocytes in diabetics are normal,<sup>22</sup> but a proportion of these cells (presumably T cells) can be shown in vitro to be sensitised against antigens derived from the substance or the hormones of the endocrine pancreas. The leucocyte-migration test (L.M.T.) was first used by NERUP et al.<sup>23</sup> to detect antipancreatic cellular hypersensitivity in human diabetes, the antigen being an extract of porcine pancreas, and the results were confirmed by the same<sup>24</sup> and other<sup>25</sup> investigators using antigens of bovine and human pancreas, respectively. These three investigations indicate that antipancreatic cellular hypersensitivity (to an antigen which is organ-specific but species-non-specific) can be displayed in between 20% and 30% of diabetics, almost all of whom have the juvenile-onset insulin-dependent type of disease. The phenomenon is present in both treated and untreated diabetics and is thus not due to exogenous-insulin therapy. The corollary to the studies with the L.M.T., which used particulate antigens, is provided by the demonstration<sup>26</sup> that soluble antigen (insulin or insulin-B-chain) can induce blastogenesis of cultured small lymphocytes from young untreated and newly diagnosed patients. But despite these findings there

is still a natural reluctance to accept the entity of autoimmune diabetes in the absence of any evidence of B-cell sensitisation—i.e., the presence in diabetic serum of a humoral autoantibody directed against the endocrine pancreas. Previous extensive searches for such an immunoglobulin had proved quite fruitless<sup>2,27,28</sup> and it was assumed not to exist; now, however, comes evidence from London<sup>29</sup> and Edinburgh (p. 1529) that antipancreatic antibody can indeed be found in diabetes—provided one looks in the right place, as it were. The combined results of BOTTAZZO et al.<sup>29</sup> and of MACCUISH and his co-workers this week indicate that this newest antibody is very similar to its counterparts in autoimmune disease; it is detectable by immunofluorescence; it is an immunoglobulin of IgG class; it fixes complement; and it probably shares the other properties common to the thyrogastric autoimmune systems. The antibody can be found in patients who have never received insulin and is directed against all cell-types in the islets of Langerhans, the exocrine pancreas being uninvolved. Thus far, islet-cell antibody has been detected only in association with overt autoimmunity, almost exclusively in insulin-dependent patients in whom diabetes coexists with single or polyglandular autoimmune disease. The simple explanation for the previous failure to detect this antibody is that the earlier searchers examined sera from unselected diabetics or deliberately excluded diabetics with overt autoimmunity.<sup>2,27,28</sup>

It will now be necessary to characterise the precise properties of islet-cell antibody, to determine the nature of the antigens against which it is directed, and to estimate its prevalence in the diabetic population. However, its discovery has two immediate implications: firstly, it lends strong credence to the concept of a true autoimmune form of diabetes mellitus, which now fulfils most of the classic criteria (close relationship with other autoimmune diseases, lymphocytic infiltration of the end-organ, circulating lymphocytes sensitised to the end-organ, circulating humoral antibody directed against the end-organ). Secondly, islet-cell antibody may prove a convenient rapid marker for identifying autoimmune diabetes, the patients thus identified being subsequently examined by other immunological techniques. The factors which initiate autoimmune diabetes remain as obscure as ever, but, having regard to all the available evidence, it may not too be fanciful to propose a unifying hypothesis whereby young subjects, who have inherited a particular complex of immune-response genes by virtue of their HL-A type, respond to (say) a viral infection by initiating an irreversible and relentless series of autoimmune processes which culminate in destruction of the endocrine pancreas and the development of clinical diabetes mellitus.

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# Cell-mediated Immunity to Human Pancreas in Diabetes Mellitus

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## SUMMARY

Cellular hypersensitivity to an extract of human pancreas, using the leucocyte migration test (LMT), was found in twenty-nine of 101 diabetic and eight of fifty normal control subjects. However, the difference in response between diabetics and controls was confined to young insulin-dependent patients, there being no distinction between normal subjects and older diabetics treated by diet or oral hypoglycemic agents. The use of rat liver mitochondria and bovine insulin as antigens in the LMT did not induce inhibition of leucocyte migration in diabetics or controls. DIABETES 23:693-97, August, 1974.

Immune phenomena in diabetes mellitus have recently been studied by in vitro tests of cell-mediated immunologic function to detect an antigen-sensitized population of lymphocytes. Thus Federlin et al.<sup>1,2</sup> showed that lymphocytes from diabetics allergic to insulin were transformed to blast cells when exposed to purified bovine insulin antigen. Other investigators, using the leucocyte migration test (LMT),<sup>3</sup> have suggested that autoimmune mechanisms may be present in diabetics by demonstrating migration inhibition of diabetic leucocytes when exposed to antigens derived from duct-ligated porcine pancreas<sup>4,6</sup> and fetal calf pancreas.<sup>5,6</sup> Attempts to inhibit migration by using insulin alone as antigen have so far been unsuccessful.<sup>4-6</sup> Migration inhibition in diabetics has also been reported using human and rat liver mitochondria;<sup>7</sup> these antigens can, however, only be considered as general nonspecific "markers" of au-

toimmune disease since inhibition of leucocyte migration to liver mitochondria is commonly present in patients with Hashimoto's thyroiditis<sup>8,9</sup> and pernicious anemia<sup>8,10</sup> as well as in primary biliary cirrhosis.<sup>8</sup>

The present study extends these observations by (1) assessing the effect of an antigen derived from human pancreas on leucocyte migration in diabetics and controls and (2) comparing the results with those obtained using rat liver mitochondria and bovine insulin as antigens.

## PATIENTS AND METHODS

*Patients.* One hundred and one diabetic and fifty-six control subjects were studied and were classified as follows:

*Young diabetics.* These were thirty-one insulin-dependent diabetics: eighteen women and thirteen men aged eighteen to forty-two years (mean 28.5) and insulin treated for between one and nineteen years (mean 7.4).

*Young controls.* These were twenty-seven healthy nondiabetic volunteers who were selected to allow statistical comparisons with the young diabetics. Fifteen were women and twelve men aged eighteen to forty-two years (mean 28.6).

*Older diabetics on oral hypoglycemic agents (OHA).* There were thirty-four diabetics diagnosed at age forty years or more who were controlled by dietary restriction plus a sulfonylurea. Twenty were women and fourteen men aged forty-five to seventy years (mean 59.7).

*Older diet-treated diabetics.* These thirty-six patients were controlled by simple carbohydrate restriction only; twenty-one were women and fifteen men aged forty-six to sixty-nine years (mean 60.5).

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**Older controls.** Twenty-three healthy nondiabetic volunteers were studied, selected by sex (thirteen women and ten men) and age (range forty-eight to sixty-nine, mean 57.4 years), to allow valid statistical comparison with the previous two groups.

**Positive reactors.** This small group, included to check the potency of the mitochondrial preparation, consisted of one patient with primary biliary cirrhosis and five with Hashimoto's thyroiditis, known to show inhibition of leucocyte migration when previously tested.<sup>9</sup>

All the diabetics regularly attended the Diabetic Department as outpatients and when blood was taken for study, were well controlled and free of infection. Control subjects were healthy volunteers, with the exception of the positive reactors.

#### Antigens

**Human pancreas** was obtained immediately after death from a previously healthy young man who sustained fatal head injuries in an automobile accident and whose kidneys were used for transplant. The fresh pancreas was cut into small pieces, homogenized in sterile phosphate buffered saline, filtered through fine gauze and then centrifuged at 700 g for twenty minutes. The supernatant was used as antigen, its protein content being adjusted to 10 mg./ml. and stored at  $-20^{\circ}\text{C}$ . in small aliquots. When required these were thawed and further diluted with tissue culture fluid consisting of Eagle's Basal Medium (EBM, Wellcome) supplemented with 10 per cent fetal calf serum. Pilot experiments showed the highest nontoxic concentration of antigen to be 200  $\mu\text{g./ml.}$ , which was used for all experiments.

**Mitochondrial preparations** were obtained from the livers of young Wistar rats by the differential centrifugation technic of Nerup and Bendixen.<sup>11</sup> This mitochondrial antigen was used at a concentration of 200  $\mu\text{g./ml.}$  culture medium.

**Highly purified bovine insulin** was obtained from the Lilly Research Laboratories (lot no. 615-D63-5) in crystalline form and diluted in culture medium to give a working concentration of 116  $\mu\text{g./ml.}$

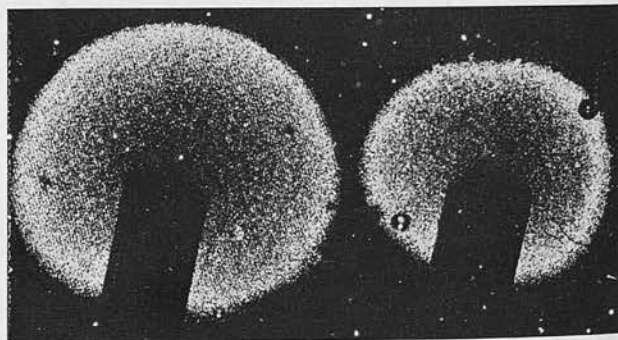
#### Leucocyte migration test (LMT)

The LMT was performed as described previously from this laboratory,<sup>9,10</sup> the method used being that of Bendixen and Soborg<sup>3</sup> with minor modifications. The theoretical basis of the test depends on the fact that lymphocytes from a sensitized individual, on contact with specific antigen, produce a soluble factor(s) which modifies cell migration. In the absence of antigen, cell migration is unaffected. Fifty milliliters of venous blood were collected from patients and con-

trols, heparinized (preservative-free heparin 10 units/ml.) and allowed to sediment at  $37^{\circ}\text{C}$ . from one to two hours. The leucocyte-rich plasma was removed, centrifuged at 150 g for ten minutes and the cell pellet washed three times in EBM. Contaminating red cells were lysed with ammonium chloride (0.83 per cent) for five minutes and the leucocyte pellet washed a further three times with EBM. The washed cells were then resuspended in EBM with 10 per cent fetal calf serum. Capillary tubes (25  $\mu\text{l.}$ ) were filled with the cell suspension, sealed at one end and centrifuged at 150 g for five minutes. The tubes were cut 1 mm. below the cell-fluid interface and the cell pellet positioned, with a dab of silicone grease, in a leucocyte migration chamber (Sterilin Ltd.). One series of at least three chambers was filled with culture medium alone and a second series with culture medium plus antigen. The chambers were sealed with glass coverslips and incubated on a flat surface at  $37^{\circ}\text{C}$ . for twenty-four hours. The fanlike pattern of migration was then projected (Projectina microscope) and the area measured by planimetry. The effect of antigen on cell migration (the "migration index") was expressed as a percentage of migration without antigen using the formula:

$$\text{Migration index} = \frac{\text{Mean migration with antigen} \times 100\%}{\text{Mean migration without antigen}}$$

A figure of less than 80 per cent was taken to indicate significant inhibition of migration and above 120 per cent, significant stimulation; this range is identical to that adopted by other investigators<sup>4,7</sup> and represents two standard deviations above and below the mean migration index shown by normal subjects to antigens in this and previous studies.<sup>9,10</sup>



Photograph:

Inhibition of leucocyte migration in the presence of pancreatic antigen. On the left, leucocytes have migrated normally from the capillary tube into culture medium; on the right, migration has been inhibited by the addition of pancreatic antigen to the culture medium.

MIGRATION INDEX (%)

FIG. 1. L  
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## RESULTS

All diabetics and normal controls were tested with human pancreatic antigen (figures 1 and 2). Seventeen of the thirty-one young diabetics showed inhibition as compared with only four of the twenty-seven young controls; the mean migration index for the former ( $77.7 \pm 2.3$  S.E.M.) was significantly lower than in the latter ( $88.3 \pm 2.1$ ). In contrast only six older diabetics on OHA and six of those on diet alone showed migration inhibition compared with two of the corresponding controls; the respective mean migration indices were  $93.4 \pm 2.8$ ,  $91.5 \pm 2.4$  and  $94.4 \pm 2.5$  and do not differ significantly. The young insulin-dependent diabetics differed significantly from all other groups, both individually and collectively ( $p < 0.01$  by the Wilcoxon test) but there were otherwise no significant differences (table 1).

The results of tests using rat liver mitochondrial antigen are shown in figure 3. The mean migration

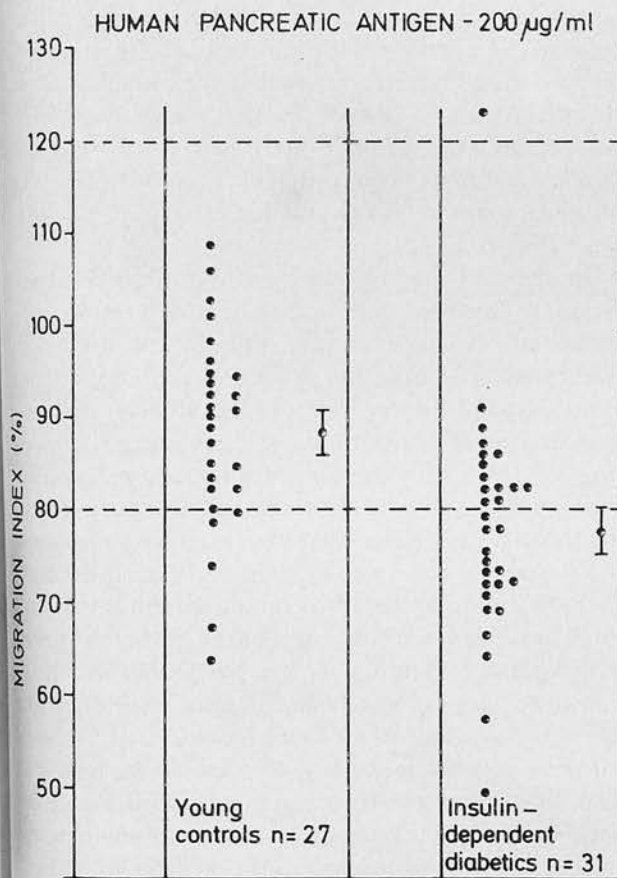


FIG. 1. Leucocyte migration tests with human pancreatic antigen in young controls and insulin-dependent diabetics. Inhibition of migration is shown by seventeen (55 per cent) diabetics compared with four (15 per cent) controls.

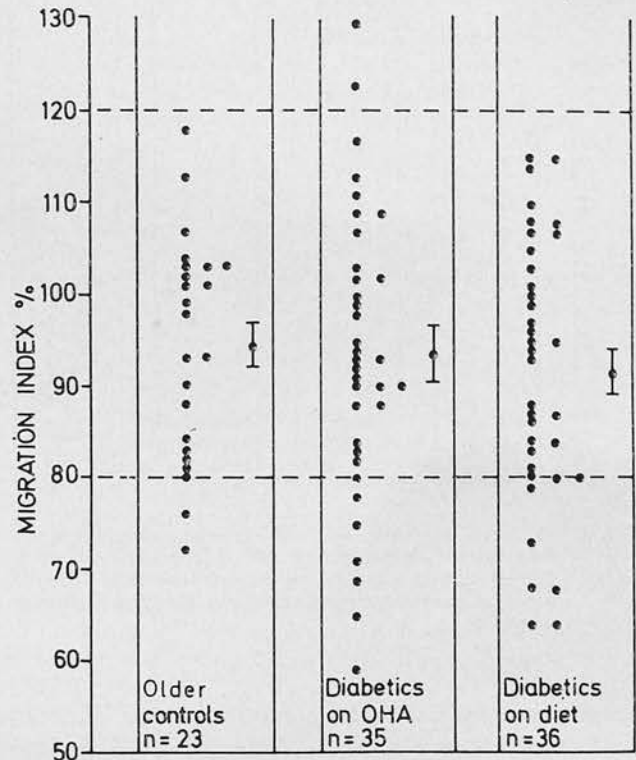
HUMAN PANCREATIC ANTIGEN - 200  $\mu\text{g}/\text{ml}$ 

FIG. 2. Leucocyte migration tests with human pancreatic antigen in older controls and insulin-independent diabetics. Inhibition of migration is shown by six (17 per cent) each of diabetics on OHA and diet respectively, compared with two (9 per cent) controls.

indices  $\pm$  S.E.M. for young diabetics, normal controls and older diabetics were  $88.5 \pm 3.2$ ,  $92.7 \pm 2.3$  and  $91.9 \pm 2.3$ , respectively, these groups not differing significantly. In contrast all patients with Hashimoto's thyroiditis or primary biliary cirrhosis, included as positive reactors, showed marked migration inhibition (mean  $69.5 \pm 2.4$ ), confirming the potency of the mitochondrial antigen; this group differed significantly from all other groups, both collectively and individually ( $p < 0.01$  by Wilcoxon's test, table 2).

Bovine insulin was used as antigen to test leucocyte

TABLE 1

Leucocyte migration tests in diabetics and controls  
Human pancreatic antigen—200  $\mu\text{g}/\text{ml}$ .

Groups compared	Difference between groups
Young diabetics—all other groups	$p < 0.01$ sig
Young controls—older controls	$p > 0.10$ NS
Young controls—older diabetics (Diet & OHA)	$p > 0.10$ NS
Older controls—older diabetics (Diet & OHA)	$p > 0.10$ NS



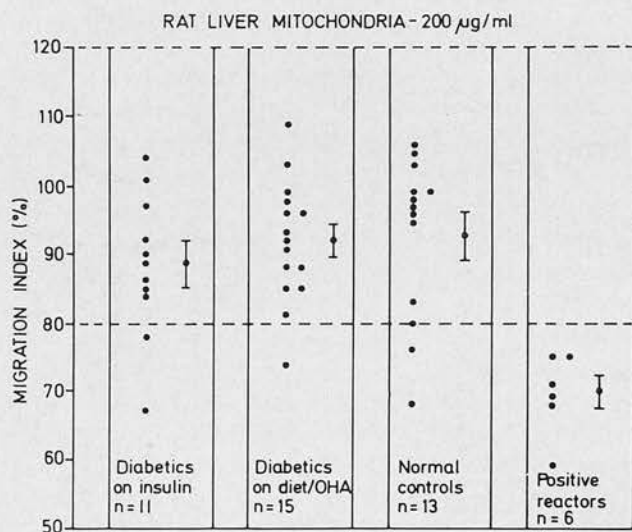


FIG. 3. Leucocyte migration tests with rat liver mitochondrial antigen in insulin-dependent and independent diabetics, normal controls and "positive reactors" (see text). Only the group of positive reactors shows significant inhibition of migration.

migration in all the young insulin-treated diabetics and corresponding controls; none showed migration inhibition to insulin at a concentration of  $116 \mu\text{g./ml.}$  culture medium (equivalent to  $3.2 \text{ i.u./ml.}$  culture medium). Above this level a toxic effect was observed. The mean migration was virtually identical in diabetics and controls ( $94.8 \pm 4.1$  and  $93.9 \pm 3.7$ , respectively).

#### DISCUSSION

Nerup et al.<sup>4</sup> first described inhibition of leucocyte migration to a pancreatic antigen in fifteen of twenty-two diabetics, all but four of whom were insulin-dependent, and most were under forty-five years of age. The antigen used was derived from pooled porcine pancreas in which atrophy of exocrine

tissue had been induced by prolonged ligation of the pancreatic duct. The same investigators<sup>5,6</sup> later used an antigen of homogenated fetal calf pancreas to demonstrate migration inhibition in thirty-one (28 per cent) of 112 diabetics. Inhibition was shown in both juvenile- and maturity-onset diabetics, occurring as often in insulin-dependent as in noninsulin-dependent patients; however, the phenomenon was most commonly found in young recently diagnosed diabetics, irrespective of therapy. The present studies using human pancreas have demonstrated migration inhibition in a comparable number of diabetics (29 per cent of 101 patients) and confirm that cell-mediated immunity as judged by the LMT is found most often in insulin-dependent patients with juvenile-onset diabetes. As yet we have not tested a sufficient number of young insulin-dependent patients, or newly diagnosed untreated diabetics, to draw any further conclusions. Nerup et al. did not find inhibition of migration in any type of diabetic by using porcine or bovine insulin antigens,<sup>4-6</sup> and this has been our experience using bovine insulin. It would seem that the results of the present and previous studies indicate the existence in diabetes of a state of cell-mediated immunity to an antigen which is present in the pancreas, species-nonspecific (demonstrable with porcine, bovine and human pancreas), and different from insulin. The phenomenon is found almost exclusively in juvenile-onset diabetics.

In diabetics the position regarding migration inhibition to antigens from other organs is more controversial. Richens et al.<sup>7</sup> showed inhibition in twenty-six of thirty-five young insulin-dependent diabetics and in four of twelve elderly insulin-independent diabetics tested with an antigen consisting of liver mitochondria from young Sprague-Dawley rats; subsequent studies by the same investigators have suggested that the antigenic component is localized to the inner mitochondrial membrane.<sup>12</sup> Richens et al. also described migration inhibition in a small number of insulin-dependent diabetics tested with human liver mitochondria but found no inhibition to antigens of mitochondria from rat kidney and adrenal.<sup>7</sup> On the other hand Nerup et al.<sup>4-6</sup> used antigens of porcine kidney, porcine liver, fetal calf liver and thymus in their series of diabetics and did not demonstrate migration inhibition to any of these antigens. Our own results, using rat liver mitochondria of proven potency, are in agreement with the latter investigators. The reason for the differences between the various studies are not clear but may in part reflect different methods of antigen preparation:

TABLE 2

Leucocyte migration tests in diabetics and controls  
Rat liver mitochondrial antigen— $200 \mu\text{g./ml.}$

Groups compared	Difference between groups
Young diabetics—controls	$p > 0.10 \text{ NS}$
Young diabetics—older diabetics (Diet & OHA)	$p > 0.10 \text{ NS}$
Older diabetics—controls (Diet & OHA)	$p > 0.10 \text{ NS}$
Positive reactors—all other groups. (Hashimoto, primary biliary)	$p < 0.01 \text{ sig}$

Richens et al. used the method of Zamecnik and Keller<sup>13</sup> and employed as antigen the fraction obtained by ultracentrifugation at 5,000 g, while the earliest study of Nerup et al.<sup>4</sup> used a different method of antigen preparation<sup>11</sup> and employed as antigen the fraction obtained by centrifugation at 104,000 g, which has a high content of microsomes.

Further studies are required to clarify this problem and also to attempt to define which component of pancreatic tissue acts as antigen toward the lymphocyte population in patients with diabetes mellitus.

#### ACKNOWLEDGMENT

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# Cell-mediated Immunity in Diabetes Mellitus

## Lymphocyte Transformation by Insulin and Insulin Fragments in Insulin-treated and Newly-diagnosed Diabetics

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### SUMMARY

Using a radioisotope labeling technic, the ability of bovine and porcine insulin antigens to induce lymphocyte transformation was tested with cells from the peripheral blood of thirty nondiabetic controls, fifty established insulin-dependent diabetics with no evidence of insulin allergy, and ten newly diagnosed diabetics (five untreated, five insulin-treated for less than three weeks). Lymphocytes from twenty-six (42 per cent) of the diabetics showed significant blastogenesis to bovine or porcine insulin, as compared with two (7 per cent) of controls; the phenomenon was shown by both established and newly diagnosed patients including four who had never received insulin. The results indicate that cellular hypersensitivity to insulin, as judged by an *in vitro* test, is relatively common in insulin-treated diabetics without *in vivo* evidence of allergy, and suggest that hypersensitivity may also be present in untreated diabetics.

Lymphocytes from twenty-one of the twenty-six diabetics who responded to intact insulin were further tested using bovine and porcine insulin A chain and bovine B chain as antigens. The A chain of either insulin induced significant blastogenesis in only one diabetic but bovine B chain induced significant blastogenesis in fourteen (67 per cent) of the patients tested. These results suggest that B chain is the major antigenic site determining cellular hypersensitivity to insulin. DIABETES 24:36-43, January, 1975.

Cell-mediated immune mechanisms in human diabetes mellitus have recently been studied by *in vitro* tests. The leucocyte migration test<sup>1</sup> has demonstrated migration inhibition of diabetic leucocytes cultured in the presence of antigens derived from porcine,<sup>2</sup> fetal calf<sup>3</sup> or human pancreas.<sup>4</sup> The phenomenon was most common in recently-diagnosed, juvenile-onset diabetics and was independent of antidiabetic treatment, being found in patients who were insulin-dependent, insulin-independent or untreated at the time of study.<sup>2-4</sup> The same investigators did not find migration inhibition

of diabetic leucocytes by antigens derived from liver, kidney, thymus or adrenal, nor did purified insulin inhibit migration; it has therefore been suggested that the results indicated the presence in young diabetics of a state of cellular immunity to species-nonspecific antigen(s), pancreatic in origin but different from insulin. The organ-specificity of the antigen must, however, remain in doubt at present in view of reports<sup>5,6</sup> that liver mitochondria can inhibit migration of leucocytes in diabetes as well as in other diseases<sup>7-9</sup> of established autoimmune etiology.

Mitogen- or antigen-induced transformation of lymphocytes to blast cells<sup>10</sup> are alternative *in vitro* tests of cellular immune function. The mitogen phytohemagglutinin (PHA) is considered to stimulate mainly T lymphocytes<sup>10,11</sup> and has been used to demonstrate that transformation responses are normal in well controlled diabetics<sup>12,14</sup> and depressed in poorly controlled diabetics.<sup>13,14</sup> In contrast there is little information concerning antigen-induced lymphocyte transformation in diabetes, but various investigators<sup>15-17</sup> have shown that bovine insulin can induce blastogenesis in lymphocytes from diabetics with both immediate (urticarial) and delayed (cutaneous) insulin allergy.

In the present investigation we have used bovine and porcine insulin as antigens to detect sensitized lymphocyte populations in treated diabetics without *in vivo* evidence of insulin allergy, and in untreated diabetics; in addition, isolated fragments (A and B

TABLE 1

Composition of Lilly bovine and porcine insulins used as antigens

Content*	Bovine Insulin	Porcine Insulin
Proinsulin	0.06	0.15
Glucagon	0.007	0.002
Zinc	0.80	0.70
Monodesamido insulin	13.0	7.0
"Arginine" insulins	1.0	2.0
Potency (U/mg.)	25.4	25.4

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chain) of the insulins from both species have been used in an attempt to localize the immunogenic portion of the insulin molecule.

#### PATIENTS AND METHODS

**Patients.** Sixty diabetics were studied. Forty-two were women and eighteen were men, from sixteen to seventy-one years of age (mean 42.1). Fifty were established diabetics and have been insulin-treated between six months and twenty-four years. Ten were newly diagnosed diabetics, five of whom had been insulin-treated for three weeks or less and five of whom were untreated at the time of study. All were attending an outpatient clinic and in all (except for the newly-diagnosed) the disorder was well controlled as judged by the following criteria: steady weight and insulin dosage; absence of thirst or polyuria; no heavy glycosuria; no ketonuria; mid-morning blood glucose less than 250 mg. per 100 ml. All were taking insulin of bovine origin, prepared either as soluble (Regular) insulin or in a sustained-release form (Protamine-Zinc or IZS Lente insulin). None had evidence of im-

TABLE 2

Ability of varying concentrations of bovine insulin to induce transformation (judged by  $^3\text{H}$ -thymidine uptake) of cultured lymphocytes from a diabetic with insulin allergy. No significant increase of blastogenesis is obtained by adding more than 10  $\mu\text{g}$  insulin per culture.

	Control*	Cultures plus Bovine Insulin Antigen*			
		50 $\mu\text{g}/\text{ml}$ .	500 $\mu\text{g}/\text{ml}$ .	1,000 $\mu\text{g}/\text{ml}$ .	5,000 $\mu\text{g}/\text{ml}$ .
		1 $\mu\text{g}/\text{culture}$	10 $\mu\text{g}/\text{culture}$	20 $\mu\text{g}/\text{culture}$	100 $\mu\text{g}/\text{culture}$
First Assay	1,080	8,720	12,480	13,640	10,390
Second Assay	1,769	9,550	14,570	14,210	12,620

\*All results as cpm

mediate or delayed-type insulin allergy.

The control group comprised thirty healthy non-diabetic volunteers. For statistical purposes it was selected to be comparable in terms of sex (twenty women, ten men) and age (range fifteen to seventy-two years, mean 40.8 years) with the diabetic group. Most controls were laboratory or hospital personnel; a few were hospital outpatients who were not known to

TABLE 3

Ability of bovine insulin, porcine insulin and PHA to induce transformation (judged by  $^3\text{H}$ -thymidine uptake) of cultured lymphocytes from thirty normal subjects. Results expressed both as counts per minute (cpm) and transformation index (T.I.—see text). All figures are means of triplicate cultures.

Age/sex	Control	Bovine Insulin		Porcine Insulin		Maximal PHA
	cpm	cpm	T.I.	cpm	T.I.	Response (cpm)
15/m	939	546	0.58	751	0.80	25,100
72/f	1,318	806	0.61	1,216	0.92	17,900
29/f	704	501	0.71	613	0.87	44,400
19/m	1,188	893	0.75	1,463	1.23	22,800
34/f	1,026	784	0.76	1,281	1.24	15,000
34/f	823	669	0.80	707	0.86	16,300
66/f	975	792	0.81	800	0.82	8,700
32/f	1,133	941	0.83	1,497	1.32	22,500
53/m	1,572	1,322	0.85	1,352	0.86	38,400
24/f	759	654	0.86	502	0.66	16,800
58/f	1,033	914	0.88	1,109	1.07	11,100
36/m	1,007	899	0.89	1,025	1.02	14,900
26/f	659	589	0.90	594	0.90	26,100
68/f	1,583	1,521	0.96	1,456	0.92	21,200
32/m	1,292	1,250	0.97	1,066	0.83	10,800
48/f	875	860	0.98	684	0.78	19,800
41/f	958	940	0.98	937	0.98	19,200
28/f	1,106	1,104	1.00	1,175	1.06	44,700
36/f	1,136	1,178	1.04	1,264	1.11	14,100
18/m	848	891	1.05	903	1.06	29,300
69/m	710	750	1.05	726	1.02	22,500
54/f	1,285	1,363	1.06	926	0.72	7,600
26/f	955	1,042	1.09	1,781	1.86	20,300
46/f	797	880	1.10	1,260	1.58	27,400
48/f	923	1,017	1.10	991	1.07	13,300
45/m	1,714	1,910	1.11	1,980	1.16	23,500
32/f	1,073	1,222	1.14	1,300	1.21	38,500
57/m	1,809	2,583	1.43	1,835	1.01	16,700
36/m	1,465	2,109	1.44	1,584	1.08	30,000
48/f	968	1,457	1.51	1,327	1.37	54,100



TABLE 4

Ability of bovine insulin, porcine insulin and PHA to induce transformation (judged by  $^3\text{H}$ -thymidine uptake) of cultured lymphocytes from fifty established insulin-dependent diabetics. Results as in table 3.

Study Number	Age/Sex	Control cpm	Bovine Insulin cpm	T.I.	Porcine Insulin cpm	T.I.	Maximal PHA Response (cpm)
1	27/m	964	705	0.73	1,039	1.08	35,900
2	50/f	1,541	1,123	0.73	1,326	0.86	17,500
3	42/f	1,495	1,146	0.85	1,053	0.80	22,400
4	40/f	809	728	0.90	802	0.98	18,700
5	31/m	1,475	1,388	0.94	1,400	0.95	26,400
6	62/f	878	843	0.96	805	0.92	24,100
7	65/f	1,096	1,071	0.98	956	0.87	30,700
8	40/f	1,167	1,172	1.00	1,006	0.86	17,600
9	59/f	1,122	1,120	1.00	1,327	1.10	20,400
10	66/f	994	1,001	1.01	1,351	1.36	18,500
11	45/m	1,226	1,270	1.04	1,164	0.95	26,400
12	38/f	915	959	1.05	1,049	1.15	25,000
13	42/m	1,518	1,597	1.05	2,173	1.43	13,900
14	25/f	728	760	1.05	881	1.21	17,400
15	48/f	1306	1,427	1.09	1,259	0.96	7,800
16	46/f	811	900	1.11	853	1.05	41,800
17	55/f	963	1,079	1.12	1,014	1.05	21,400
18	20/f	870	979	1.13	1,357	1.56	33,300
19	37/f	931	1,051	1.13	863	0.93	27,600
20	69/m	1,071	1210	1.13	1,089	1.02	10,600
21	23/f	1,000	1,140	1.14	1,320	1.32	19,300
22	46/m	939	1,067	1.14	1,221	1.30	27,400
23	41/f	1043	1,210	1.16	1,079	1.03	12,700
24	45/f	720	848	1.18	794	1.10	15,500
25	21/f	923	1,155	1.25	1,609	1.74	50,600
26	70/f	1,320	1,690	1.28	1,990	1.51	20,400
27	25/f	1,448	1,853	1.28	1,445	1.00	26,600
28	62/m	962	1,228	1.28	1,062	1.11	38,200
29	14/f	634	818	1.29	799	1.26	8,500
30	27/f	859	1,112	1.30	1,120	1.30	11,900
31	22/m	951	1,109	1.31	1,561	1.83	21,100
32	67/f	869	1,169	1.34	927	1.07	21,200
33	57/m	1,446	1,952	1.35	2,053	1.42	39,500
34	52/f	1,094	1,557	1.42	1,503	1.37	14,000
35	46/m	832	1,192	1.43	1,278	1.53	10,300
36	42/f	1,391	2,130	1.53	2,465	1.77	18,200
37	30/f	989	1,533	1.55	1,553	1.57	35,900
38	41/f	943	1,525	1.62	1,719	1.82	19,200
39	37/f	885	1,430	1.62	1,686	1.90	27,900
40	48/f	1,000	1,659	1.66	1,809	1.81	26,700
41	61/f	1,832	3,077	1.68	2,994	1.63	18,900
42	54/f	1,016	1,802	1.77	1,641	1.61	20,500
43	59/f	1,547	2,769	1.79	2,614	1.69	14,100
44	52/f	843	1,512	1.79	2,366	2.80	17,600
45	61/m	1,042	1,963	1.88	1,777	1.71	26,000
46	62/f	897	1,790	1.99	1,282	1.42	47,100
47	26/f	734	1,508	2.06	1,454	1.98	21,300
48	71/f	1,458	3,164	2.17	3,762	2.58	41,000
49	38/m	864	2,449	2.83	2,049	2.38	13,900
50	39/f	1,046	3,797	3.63	4,707	4.50	34,500

have endocrine or immunological disease.

**Antigens.** Bovine and porcine insulins of known potency and composition (table 1) were obtained from the Lilly Research Laboratories. Bovine insulin A chain and B chain (aminoethylated), and porcine insulin A chain (S-sulfonate) were obtained from the same source. All preparations were in crystalline powder form. On the day of use they were dissolved in a

mixture of 10 per cent sterile phosphate buffered saline/80 per cent Eagle's Basal Medium/10 per cent fetal calf serum.

The optimal concentration of insulin for use in our investigations was determined by measuring the blastogenic effect of insulin on lymphocytes from a diabetic with known insulin allergy; the patient was a twenty-two-year-old man who had a daily insulin re-

TABLE 5

Ability of bovine insulin, porcine insulin and PHA to induce transformation (judged by  $^3\text{H}$ -thymidine uptake) of cultured lymphocytes from ten newly diagnosed diabetics. Results as in table 3.

Study Number	Age/Sex	Control	Bovine Insulin		Porcine Insulin		Maximal PHA Response (cpm)
		cpm	cpm	T.I.	cpm	T.I.	
51	58/f	1,505	1,472	0.98	1,430	0.95	17,700
52	37/m	841	832	0.99	699	0.83	7,800
53	19/m	1,043	1,029	0.99	1,343	1.29	19,400
54	42/m	891	972	1.09	881	0.99	9,900
55	18/m	619	680	1.10	930	1.50	13,800
56	19/f	1,473	2,458	1.67	2,591	1.76	38,000
57	23/f	757	1,498	1.98	1,520	2.00	25,400
58	30/m	802	1,626	2.03	1,729	2.16	16,500
59	16/m	1,138	2,675	2.35	2,281	2.00	25,400
60	19/f	1,307	5,313	4.07	5,925	4.53	16,600

quirement in excess of 200 units in addition to cutaneous reactions to bovine insulin injections, and who therefore showed evidence of both humoral and cell-mediated insulin hypersensitivity. Lymphocytes were obtained from this patient on two separate occasions, prepared and cultured as described below, and exposed to varying concentrations of bovine insulin antigen. The results (table 2) show that maximal blastogenesis was stimulated with insulin at a concentration of 500  $\mu\text{g}/\text{ml}$ . (10  $\mu\text{g}/\text{culture}$ ), no significant increase in lymphocyte  $^3\text{H}$ -thymidine uptake being induced by higher concentrations of insulin. Thus on the basis of this preliminary dose-response analysis, all experiments were performed with solutions containing 500  $\mu\text{g}/\text{ml}$ . intact insulin or insulin fragment.

**Sample Collection and Lymphocyte Culture.** Fifteen milliliters venous blood was removed from each patient and control at mid-morning and anticoagulated with preservative-free heparin. Lymphocytes were prepared from whole blood by the methods (density centrifugation, repeated washing in EBM) described in previous studies<sup>14,18</sup> from this laboratory, and suspended in EBM with 10 per cent fetal calf serum at a concentration of  $1 \times 10^6$  cells per milliliter.

TABLE 6

Effects of bovine and porcine insulin on PHA-induced transformation of lymphocytes from five normal subjects. Neither insulin has a significant effect on the  $^3\text{H}$ -thymidine uptake of PHA-stimulated lymphocytes.

Age/Sex	Maximal $^3\text{H}$ -thymidine Uptake (cpm)		
	With PHA	PHA+B.I.*	PHA+P.I.*
36/m	32,000	35,000	31,000
19/m	25,000	24,000	22,000
32/m	9,000	8,000	9,000
26/f	28,000	26,000	26,000
36/m	14,000	16,000	14,000

\*B.I.—Bovine insulin 10  $\mu\text{g}/\text{culture}$

P.I.—Porcine insulin 10  $\mu\text{g}/\text{culture}$

A portion of the lymphocytes from each subject was cultured in the presence of the mitogen phytohemagglutinin (PHA) to provide a convenient assessment of the adequacy of general cell-mediated immune func-

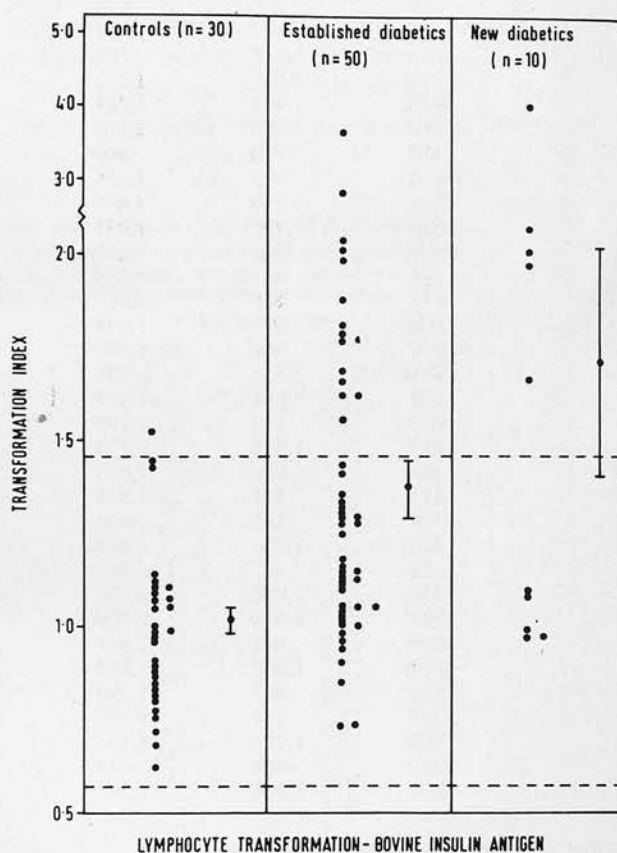


FIG. 1. Lymphocyte transformation in controls and diabetics induced by bovine insulin antigen. The mean transformation index  $\pm$  S.E.M. is shown to the right of the individual results for each group. Dotted lines (---) indicate the normal range (mean  $\pm$  2 S.D.) of transformation indices in control subjects. Significant transformation (index  $>1.45$ ) is shown by fifteen established and five newly diagnosed diabetics as compared to one control. Both diabetic groups differ significantly ( $p < 0.01$ ) from the control group.

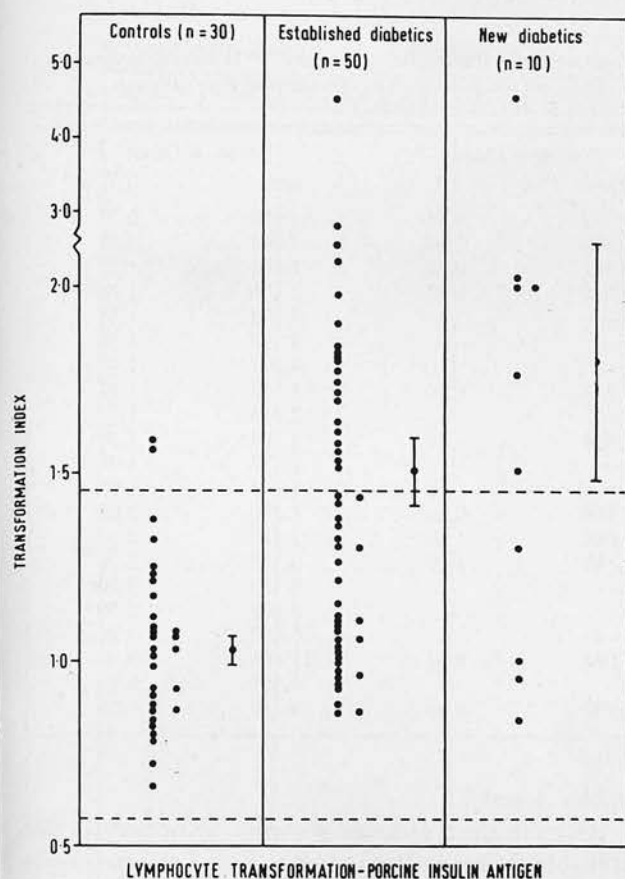


FIG. 2. Lymphocyte transformation in controls and diabetics induced by porcine insulin antigen. Symbols as in figure 1. Significant transformation is shown by twenty established and six newly diagnosed diabetics as compared to two controls. Both diabetic groups differ significantly ( $p < 0.01$ ) from the control group.

tion. The lymphocyte transformation response to PHA was measured by an extensively investigated micromethod,<sup>19</sup> which utilizes the cellular uptake of radioactive DNA precursor ( $^3\text{H}$ -thymidine) as the index of lymphocyte stimulation and has been used in our previous study<sup>14</sup> of PHA responses in diabetes mellitus. This micromethod enables a mitogen dose-response curve to be constructed for lymphocytes from each subject by using PHA at concentrations of 0.32, 0.63 and 1.25  $\mu\text{l}$  per milliliter culture. The radioactivity of stimulated cells was determined by an automatic beta counter (Nuclear Enterprises NE8312) and results are expressed as counts per minute (cpm).

The remaining lymphocytes from each patient and control were cultured in the presence of insulin antigen. The technic used was modified from the PHA micromethod<sup>19</sup> and is briefly summarized as follows: aliquots of  $2 \times 10^5$  lymphocytes were pipetted into the wells of plastic tissue culture plates (Cooke Microtiter). All experiments were performed in triplicate

and consisted of a control row of cultures without antigen, and rows to which 20  $\mu\text{l}$  of the required antigen solution (i.e. 10  $\mu\text{g}$  insulin or insulin fragment) was added. The plates were gassed with a 95 per cent air/5 per cent  $\text{CO}_2$  atmosphere and incubated at  $37^\circ\text{C}$ . in sealed, humidified containers. After five days the cultures were labeled with  $^3\text{H}$ -thymidine (specific activity 5 Ci/mmol, dose 0.4  $\mu\text{Ci}$  per culture) and terminated sixteen hours later by harvesting the cells onto glassfibre filter discs and preparing them<sup>19</sup> for scintillation counting. The prepared samples were counted in an automatic beta counter.

Results were obtained as counts per minute (cpm); the effect of antigen in stimulating blastogenesis (the 'transformation index') was expressed by the formula:

$$\text{Transformation Index} = \frac{\text{cpm of cultures with antigen}}{\text{cpm of cultures without antigen}}$$

The normal range of transformation indices using intact insulin antigens was obtained from the mean transformation index  $\pm 2$  standard deviations of the results given by both insulins in the control group (i.e. 60 experiments). The range in normals by this method was found to be 0.57-1.45 (mean  $1.01 \pm 0.44$ ), and a transformation index above 1.45 was thus taken to indicate significant antigenic stimulation of blastogenesis. Several cultures from controls and diabetics were examined by light microscopy to confirm that the uptake of radioactive label was paralleled by morphological blast transformation.

All diabetics and controls were tested with intact bovine and porcine insulin antigens. Lymphocytes from those patients who showed significant transformation were further tested with bovine A and B chain and porcine A chain.

## RESULTS

**Mitogenic effects of PHA.** The maximal PHA response of diabetics and controls (i.e. the highest lymphocyte incorporation of  $^3\text{H}$ -thymidine label induced by one of the three concentrations of PHA used) is shown in tables 3-5. The mean maximal PHA response in the thirty controls was  $23.1 \pm 4.3$  (expressed as  $\text{cpm} \times 10^3 \pm \text{S.E.M.}$ ) while the corresponding figure in the sixty diabetics was  $22.5 \pm 4.7$ . The two groups do not differ significantly in PHA responsiveness and the results confirm our earlier observation<sup>14</sup> that mitogen-induced lymphocyte transformation is normal in diabetes mellitus, except at times of severe metabolic decompensation.

The maximal PHA response of five control subjects was further measured in lymphocyte cultures to which



TABLE 7

Ability of bovine insulin A chain, porcine insulin A chain and bovine B chain to induce transformation (judged by  $^3\text{H}$ -thymidine uptake) of cultured lymphocytes from twenty-one diabetics who showed significant transformation to intact insulin molecule.

\*Study numbers identify patients in tables 4 and 5. Results as in table 3.

Study Number	Control	Bovine A Chain		Porcine A Chain		Bovine B Chain	
	cpm	cpm	T.I.	cpm	T.I.	cpm	T.I.
49	970	942	0.97	739	0.76	680	0.70
47	1,004	1,104	1.10	1,670	1.69	944	0.94
38	1,150	1,220	1.06	781	0.68	1,380	1.20
*58	902	939	1.04	747	0.83	1,136	1.26
41	1,623	1,445	0.89	—	—	2,173	1.34
45	1,684	1,161	0.69	—	—	2,339	1.39
36	1,092	963	0.88	—	—	1,527	1.40
40	870	938	1.08	523	0.60	1,313	1.51
*60	1,422	1,466	1.03	—	—	2,415	1.70
44	759	639	0.84	834	1.10	1,364	1.80
43	1,670	1,385	0.83	—	—	3,173	1.90
*57	518	682	1.10	—	—	1,171	1.90
42	925	1,833	1.98	906	0.98	1,880	2.03
*59	1,062	1,010	0.95	1,094	1.03	2,123	2.10
26	1,566	1,598	1.02	1,690	1.08	3,445	2.20
*56	1,241	1,450	1.17	—	—	6,452	5.20
39	950	1,020	1.07	—	—	5,605	5.90
37	860	1,039	1.20	—	—	6,192	7.20
48	1,287	1,364	1.06	1,094	0.85	11,866	8.70
25	705	750	1.05	—	—	6,201	8.80
46	964	972	1.01	630	0.66	9,350	9.70

\*Newly diagnosed patient

10  $\mu\text{g}$  bovine or porcine insulin had been added. The results (table 6) indicate that neither bovine nor porcine insulin have an appreciable effect on  $^3\text{H}$ -thymidine uptake in the presence of PHA when added to forty-eight-hour cultures in the stated concentrations.

*Antigenic effects of intact insulins.* The effects of bovine and porcine insulin antigens in inducing blastogenesis of lymphocytes from controls and diabetics are presented in tables 3-5 (as absolute cpm) and in figures 1 and 2. Only one normal subject showed significant transformation using bovine insulin antigen; in contrast fifteen established and five newly diagnosed diabetics (three untreated, two insulin-treated for less than three weeks) showed significant transformation, their indices ranging from 1.53 to 4.07 (figure 1). The groups of both established and newly diagnosed diabetics differed significantly from the control group ( $p < 0.01$  by Wilcoxon's test).

Experiments using porcine insulin as antigen (figure 2) gave a virtually identical pattern. Two control subjects showed significant transformation, as compared with twenty established and six newly diagnosed diabetics (four untreated, two treated for less than three weeks). Both diabetic groups again differed significantly ( $p < 0.01$ ) from the controls. Almost all the diabetics who showed transformation to porcine insulin also showed transformation to bovine insulin

(tables 4 and 5).

*Antigenic effects of A and B chains.* Lymphocytes were available for study from twenty-one of the twenty-six diabetics who showed significant transformation to intact insulin molecule. The effects of bovine A chain, porcine A chain and bovine B chain on these lymphocytes are shown in table 7 and figure 3. The A chain of either insulin was almost without effect, inducing significant blastogenesis in only one diabetic; in contrast the B chain of bovine insulin induced significant lymphocyte transformation in fourteen (67 per cent) of the twenty-one patients tested. In some diabetics the isolated B chain appeared to be more potent in stimulating blastogenesis than intact insulin molecule, the transformation index being greater than 4.0 in six patients. Statistical analysis confirmed that the effects of B chain on lymphocyte transformation differed significantly ( $p < 0.01$ ) from those of the A chain of either insulin.

#### DISCUSSION

Under the stated culture conditions, intact insulin (bovine and porcine) was observed to induce transformation of lymphocytes in vitro from one-third of the diabetics tested. In this context, insulin may be regarded as showing cellular antigenic activity analogous to the effects of PPD on lymphocytes from tuberculin-sensitized patients,<sup>20</sup> intrinsic factor on



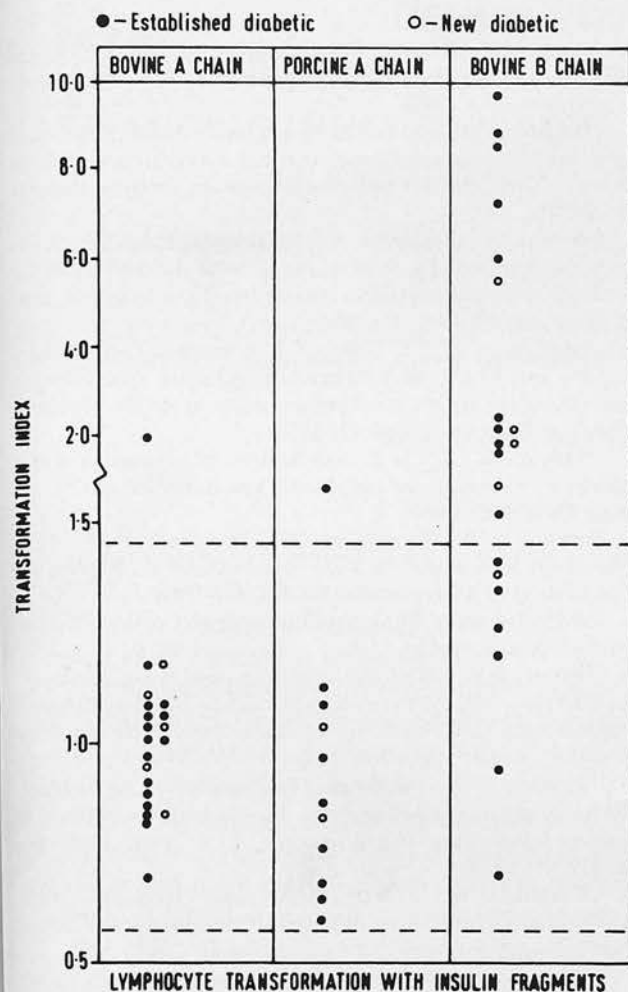


FIG. 3. Lymphocyte transformation induced by bovine and porcine A chain and bovine B chain in twenty-one diabetics who responded to intact insulin. Symbols as in figure 1. Closed circles (●)—established diabetics; open circles (○)—newly diagnosed diabetics. Significant transformation to either A chain is shown by only one diabetic, as compared to fourteen (67 per cent) who respond to B chain. The effects of B chain differ significantly ( $p < 0.01$ ) from those of the A chain of either insulin.

lymphocytes from patients with pernicious anemia<sup>21</sup> and thyroglobulin on lymphocytes from patients with Hashimoto thyroiditis.<sup>22</sup> The blastogenic effect of insulin is perhaps not surprising in the group of established diabetics that we tested: all had been insulin-treated for months or years, virtually all<sup>23</sup> would be expected to have humoral antibodies to injected insulin, and it is reasonable to suppose that some have developed cellular immunity in the form of an insulin-sensitized lymphocyte subpopulation. Similar findings were reported by Halpern et al.<sup>15</sup> and Federlin et al.<sup>16,17</sup> in diabetics with insulin allergy, both of immediate (urticarial) and delayed (cutaneous) type; our own experience suggests that the phenomenon is

not uncommon in diabetics who have no clinical signs of allergy. In these patients the *in vitro* test of lymphocyte function may be the most sensitive measurement of an insulin hypersensitivity which is not of clinical significance.

More interesting is the finding of insulin-induced lymphocyte transformation in six of ten newly diagnosed patients, four of whom had never been given insulin and two of whom had been insulin-treated for less than three weeks. Further studies are needed to confirm these observations in larger numbers of untreated diabetics but the present results at least suggest that a proportion of such patients have an insulin-sensitized lymphocyte population before exogenous insulin has been given and before<sup>23</sup> humoral insulin antibodies have developed. Nondiabetics do not show this cell population and its existence may reflect a state of cell-mediated autoimmunity, to insulin or insulin precursor, in early diabetes.

The virtually identical findings with bovine and porcine insulin prompted us to examine their component chains for antigenicity, and the results show a clear-cut difference between the effects of the two major chains. On the one hand, neither bovine nor porcine A chain induced significant lymphocyte transformation, and as the structure of human insulin A chain is identical to that of porcine insulin<sup>24</sup> it may be presumed that human A chain is also without blastogenic effect. On the other hand, bovine insulin B chain (which is identical to that of porcine insulin and differs<sup>24</sup> from the amino-acid sequence of human B chain only at position 30) had a striking effect in inducing blastogenesis of lymphocytes from two thirds of those patients who responded to intact insulin. The results thus suggest that B chain is the major antigenic site producing cellular hypersensitivity to insulin. Support for this hypothesis is given by the *in vivo* animal experiments of Clark and Munoz, who injected guinea pigs with bovine insulin, A and B chain in Freund's adjuvant: Cutaneous hypersensitivity was readily elicited by intact insulin and B chain, but A chain had no significant effect.<sup>25</sup>

The role of insulin B chain in cell-mediated immunity to insulin may be contrasted with the humoral response to insulin, where various studies<sup>26,27</sup> have suggested that the A chain is the major determinant of antibody production. It seems that differing antigenic activity, whether cellular or humoral, may reside at differing sites on the insulin molecule, such activity being perhaps governed partly by genetically determined configuration of antibody binding sites<sup>28</sup> and partly by the relationship between the structure of

antibody and the amino-acid sequence against which it is directed. Finally, the apparent lack of antigenicity of A chain in our experiments might be explained by the physical changes undergone by this peptide during the splitting of insulin: Isolated A chain is 'stretched' by comparison with its configuration in the intact insulin molecule,<sup>29</sup> and the intra-chain disulfide bridge between six and eleven is broken with the formation of cysteic acid residues<sup>26</sup> which could prevent antigen-antibody reaction at this site.

Further studies should attempt to delineate more clearly the cellular antigenic portion of insulin and to indicate whether related proteins or precursors (e.g. proinsulin, C-peptide) have similar properties in early diabetes.

#### ACKNOWLEDGMENT

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## TECHNIQUES

# A RAPID MICRO-METHOD FOR THE PHYTO-HAEMAGGLUTININ-INDUCED HUMAN LYMPHOCYTE TRANSFORMATION TEST

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## SUMMARY

A quantitative *in vitro* method for phytohaemagglutinin (PHA) induced lymphocyte transformation is described. This method has the following advantages.

Cultures require as few as  $10^5$  lymphocytes and are performed in microtrays, over a short incubation period (48 hr).

A short pulse time of 4 hr with [ $^3\text{H}$ ]thymidine is utilized. This enables high levels of [ $^3\text{H}$ ]thymidine to be maintained throughout the labelling period, ensures maximum incorporation of thymidine into cellular DNA, and diminishes cellular damage by internal irradiation.

The extraction process has been simplified by using a communal washing procedure after drying the cultures on glass-fibre filter discs. The procedure is both quicker and more reproducible (coefficient of variation 6%) than extraction and drying on filtration manifolds (coefficient of variation 23%).

The effect of adjusting variables such as the number of cells, incubation time, concentration of [ $^3\text{H}$ ]thymidine, concentration of PHA, specific activity of [ $^3\text{H}$ ]thymidine and duration of [ $^3\text{H}$ ]thymidine pulse has been studied in order to approach optimal labelling conditions for the assay system.

The reproducibility of the method has been investigated by repeated testing of normal individuals on a day-to-day basis and over extended periods. The mean coefficient of variation for samples repeated daily and over longer time intervals (weeks or months between samples) was 15%.

## INTRODUCTION

The transformation of cultured small lymphocytes by the mitogen phytohaemagglutinin (PHA) is now extensively employed as a general assessment of cell-mediated immune function in a variety of clinical states (Oppenheim, Blaese & Waldmann, 1970; Gatti, Garrioch & Good, 1970; Trubowitz, Masek & Del Rosario, 1966; Lischner, Punnet & DiGeorge,

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1967; Hosking, Fitzgerald & Simons, 1971). The most frequently used method of quantifying the response to PHA is by measuring the rate of DNA synthesis, using the incorporation of a labelled precursor such as [ $^3\text{H}$ ]thymidine.

This procedure, as generally applied in the investigation of clinical disease, has suffered from a number of disadvantages.

(1) Often there has been inadequate definition of the dose-response relationship of PHA with comparisons between groups limited to a single PHA dose.

(2) The majority of assay systems described have used  $10^6$  or more lymphocytes per culture, thus requiring substantial volumes of blood.

(3) A lengthy culture period, often of 72 hr, has been adopted.

(4) The extraction methods employed are frequently elaborate and time-consuming, involving repeated washing of cells either by centrifugation or filtration.

(5) The culture conditions are often not optimal for maximum incorporation of label, thus reducing sensitivity and discrimination of the assay.

These factors have probably contributed to the conflicting reports from different workers of the responsiveness of lymphocytes to PHA in immunological deficiency (Gotoff, 1968) and certain forms of neoplasia (Han & Takita, 1972; Paty & Bone, 1973) as well as in other clinical states.

We describe here an *in vitro* lymphocyte transformation method which attempts to overcome these difficulties; cell numbers per culture have been reduced, the DNA extraction method is simple and highly reproducible, the conditions of culture have been adjusted to ensure optimal labelling of DNA, and a complete dose-response profile has been established.

## MATERIALS AND METHODS

### Materials

*Phytohaemagglutinin (PHA)*. Bacto PHA-P (Difco Laboratories) of the same batch (number 575219) was used throughout. It was reconstituted as recommended by the manufacturers with distilled water, stored in 0.10 ml aliquots at  $-20^\circ\text{C}$  and used immediately once thawed.

*Eagle's basal medium (EBM)*. This contained antibiotics and was buffered with 0.1% bicarbonate (TC30, Wellcome Reagents Ltd).

*Foetal calf serum*. CS06, Wellcome Reagents Ltd.

*Tritiated thymidine*. [ $^3\text{H}$ ]Thymidine was obtained from the Radiochemical Centre, Amersham. Two specific activities were used: 5 Ci/mmol (Code TRA 120); and 15 Ci/mmol (Code TRK 120).

*Scintillation fluid*. NE233, Nuclear Enterprises Ltd.

*Glassfibre filter discs*. Whatman GF/C, diameter 2.5 cm, marked with typeface.

*Ficoll*. Sigma Chemical Company.

*Triosil 440*. Nyegaard & Co., Norway.

### Collection of blood samples and separation of lymphocytes

Venous blood (10–20 ml/subject) was obtained from a panel of healthy laboratory personnel with preservative-free heparin as anticoagulant (Evans Medical, 100 units/ml blood). The blood was layered onto an equal volume of Ficoll-Triosil (specific gravity 1.076) and



fractionated by density gradient centrifugation (Perper, Zee & Mickelson, 1968). The lymphocyte layer was removed, the cells washed three times in EBM and finally resuspended in EBM supplemented with 10% foetal calf serum. Cell suspensions were counted and adjusted to the required concentration; all suspensions contained more than 95% lymphocytes with a viability greater than 98% when tested by the Trypan Blue dye exclusion method.

#### *General procedure for lymphocyte culture*

Stock PHA was diluted in EBM, with 10% foetal calf serum, to the appropriate concentration for each particular experiment. Aliquots of 20  $\mu$ l of diluted mitogen were pipetted into the wells of Cooke Microtitre plastic culture plates (M29ART, Flow Laboratories), and 200  $\mu$ l of lymphocyte suspension (containing  $0.2 \times 10^6$  cells) added to each well. All determinations were performed in triplicate and included control cultures without PHA. The plates were incubated in a humidified 5% CO<sub>2</sub>:95% air atmosphere for 44 hr; [<sup>3</sup>H]-thymidine was then added and incubation continued for a further 4 hr.

#### *Variations in culture conditions in individual experiments*

In experiment 1, the response of  $0.2 \times 10^6$  cells per culture to a range of six concentrations of PHA (from 0.16 to 5.0  $\mu$ l/ml culture) was investigated. Each culture received 0.4  $\mu$ Ci of [<sup>3</sup>H]thymidine (5 Ci/mmole) and the incorporation of label was measured after 4 hr.

In experiment 2, the effect of incubation time was investigated using  $0.2 \times 10^6$  lymphocytes per culture and PHA at a concentration of 1.25  $\mu$ l/ml. Cultures were incubated for periods ranging from 4 to 144 hr, followed in each case by a 4-hr pulse with 0.4  $\mu$ Ci of [<sup>3</sup>H]-thymidine (5 Ci/mmole).

In experiment 3, the optimum concentration of thymidine per culture was investigated. Cell numbers and PHA concentration were as for experiment 2 and a constant incubation time of 44 hr was used. [<sup>3</sup>H]thymidine was then added in final concentrations varying from  $2 \times 10^{-4}$  mM to  $16 \times 10^{-4}$  mM and incubation continued for a further 4 hr.

In experiment 4, the effect of increasing specific activity of [<sup>3</sup>H]thymidine was investigated. Culture conditions were as for experiment 3 but a constant concentration of [<sup>3</sup>H]-thymidine ( $32 \times 10^{-4}$  mM) was used, and the specific activity varied from 10–0.16 Ci/mmole.

In experiment 5, the length of time over which the rate of incorporation of label remained maximal was investigated by both continuous and short-term pulsing. Culture conditions were as for experiment 3 but each culture received a constant dose of 0.4  $\mu$ Ci of [<sup>3</sup>H]-thymidine, of specific activity 5 Ci/mmole and the duration of thymidine pulse was varied from 4 to 24 hr. Subsequent experiments, using lymphocytes from the same pool of normal donors and identical culture conditions, compared the effects of both hourly and continuous pulsing, with a constant dose of 0.8  $\mu$ Ci of [<sup>3</sup>H]thymidine per culture over a period of 4 hr.

In experiment 6, the concentration of cell suspensions was varied from  $1 \times 10^5$ /ml to  $3 \times 10^6$ /ml (i.e.  $0.2 \times 10^5$ – $0.6 \times 10^6$  cells/culture). All cultures were stimulated with PHA at 1.25  $\mu$ l/ml, incubated for 44 hr and labelled with 0.4  $\mu$ Ci of [<sup>3</sup>H]thymidine for a further 4 hr.

In experiment 7, where several aspects of reproducibility were examined, the following culture conditions were used:  $1 \times 10^6$  cells/ml ( $2 \times 10^5$ /culture); PHA at concentrations of

0.32, 0.63 and 1.25  $\mu\text{l/ml}$  culture; [ $^3\text{H}$ ]thymidine of specific activity 5 Ci/mmmole at a concentration of 4  $\mu\text{Ci/culture}$ ; incubation time of 44 hr; [ $^3\text{H}$ ]thymidine pulse of 4 hr.

#### General procedure for culture extraction

At the end of the culture period, the cells in each well were resuspended by gentle pipetting and transferred to numbered glass-fibre filter discs which had previously been placed on a pin board. The discs were then dried either on the bench with the aid of a fan (for approximately 1 hr) or by placing the pin board in an incubator or hot-air oven (for approximately 20 min). Dried discs were collected together in a large flask and subjected to a series of communal rinses using successively 5% trichloroacetic acid, phosphate-buffered saline (pH 7.2) and absolute methanol, each rinse lasting for 5 min. The discs were then redried in an oven on sheets of absorbent paper, placed in glass counting vials containing 5 ml of scintillation fluid and finally counted for 60 sec in an automatic beta counter (Packard Tricarb 2425). Results are expressed as counts per minute (cpm), the counting efficiency being constant.

Vials and scintillation fluid were re-usable by simply removing the filter disc. Before re-use the vials were screened by counting for 10 sec and any giving a background of greater than 100 cpm were rejected.

#### Variations in culture extraction procedure

In experiment 7, the reproducibility of the method described above, that is the 'dry and communal rinse' procedure, was compared with the more commonly used method of harvesting and extraction on a filtration manifold: after culture in micro plates under standard conditions, cells were bulked together and aliquots distributed on a series of filter discs, which were then washed either on a filtration manifold or by the 'communal rinse' method.

## RESULTS

### (1) Dose response to PHA

Fig. 1 shows the mean response of lymphocytes from eight subjects to a range of six concentrations of PHA. A good level of [ $^3\text{H}$ ]thymidine incorporation was consistently achieved at all doses, the peak response lying between 0.32 and 1.25  $\mu\text{l}$  of PHA per ml culture. Fig. 1 also illustrates the enhanced uptake of label obtained when the optimal concentration of thymidine is used (*vide infra*). Maximal levels of incorporation again occurred at PHA doses between 0.32 and 1.25  $\mu\text{l/ml}$  culture; at higher doses the toxic effect of PHA was again evident and the uptake of label was less.

### (2) Effect of incubation time on [ $^3\text{H}$ ]thymidine incorporation

Lymphocytes from five subjects were used. Fig. 2 shows that under the stated conditions, the highest incorporation of label was achieved with a total culture period of 48 hr, was slightly lower after 72 hr and thereafter declined rapidly with increasing length of culture. Thus, the period arbitrarily chosen for convenience, i.e. 48 hr, is that which also yields the highest observed incorporation.

### (3) Relationship between thymidine concentration and incorporation of [ $^3\text{H}$ ]thymidine

Lymphocytes from five donors were used. The uptake of [ $^3\text{H}$ ]thymidine in stimulated

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cells was found to occur in linear fashion until the concentration of thymidine reached  $8 \times 10^{-4}$  mM ( $4 \mu\text{Ci/ml}$ ) (Fig. 3). Further increase in thymidine concentration thereafter did not lead to a corresponding increase in incorporation of label, indicating that the saturating level of isotope (i.e. the concentration at which the influence of endogenous thymidine is minimal in producing a dilutional effect on the specific activity of the label) was reached

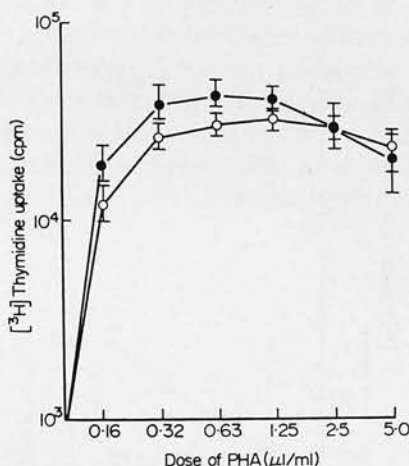


FIG. 1. Mean lymphocyte responses in eight subjects to a range of PHA doses. (○) Mean  $\pm$  s.e.m. of response at a sub-optimal concentration of thymidine ( $4 \times 10^{-4}$  mM). (●) Mean  $\pm$  s.e.m. of the responses under conditions of thymidine saturation (concentration  $16 \times 10^{-4}$  mM).

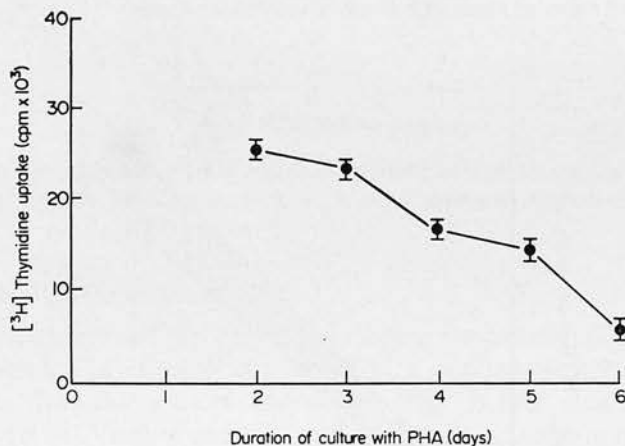


FIG. 2. Effect of length of culture on the level of [ $^3\text{H}$ ]thymidine incorporation of PHA-stimulated lymphocytes from five subjects. Each point represents the mean  $\pm$  s.e.m. of five experiments.

at  $8 \times 10^{-4}$  mM under the stated conditions. In unstimulated cultures, the amount of radiation increased steadily throughout the range of thymidine concentration, reflecting the increasing background level of radiation.

#### Effect of increasing the specific activity of [ $^3\text{H}$ ]thymidine

This experiment used lymphocytes from six donors and enabled the toxic effects of [ $^3\text{H}$ ]

thymidine label caused by internal radiation to be investigated. Fig. 4 demonstrates that a linear incorporation of label was obtained throughout the range of specific activities used, suggesting that even at the higher levels of radiation DNA synthesis was not adversely affected. However, when the actual incorporation at each specific activity was compared with that predicted by calculation from the incorporation at the lowest specific activity

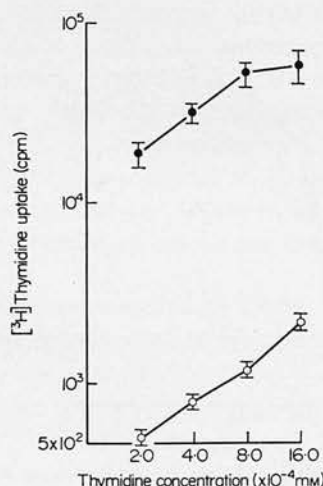


FIG. 3. The relationship between thymidine concentration and incorporation of label in lymphocytes from five subjects. (●) Mean  $\pm$  s.e.m. of five experiments using PHA-stimulated cells. (○) Mean  $\pm$  s.e.m. of identical cultures of unstimulated cells.

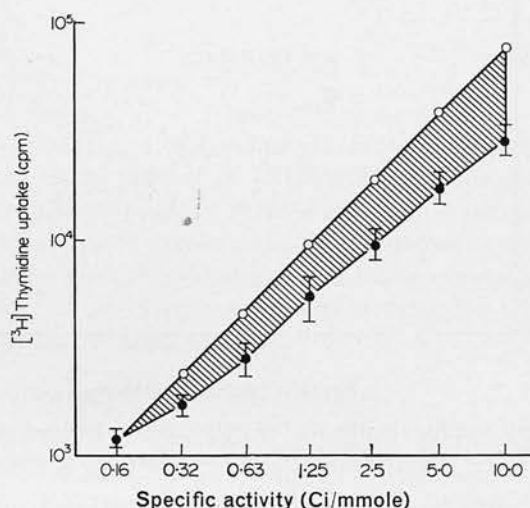


FIG. 4. Relationship of specific activity of [ $^3\text{H}$ ]thymidine and incorporation of label in lymphocytes from six subjects. (●) Mean  $\pm$  s.e.m. of the observed cpm at each specific activity. (○) Value expected at each specific activity by calculation from the cpm observed at a specific activity of 0.16 Ci/mmole (where irradiation damage is minimal). The hatched area shows the difference between expected and observed values, indicating a loss of cpm due to irradiation damage.



(where toxic effects could be assumed to be negligible) it was clear that a progressive decline in incorporation from the calculated level was taking place. Since the concentration of thymidine was at saturating levels throughout the range of specific activities this loss could be attributed to the effect of internal irradiation due to the increase of specific activity alone. Thus it is evident that the use of higher specific activities of isotope, whilst increasing the cpm, also incurs the penalty of a progressive burden of damage due to internal irradiation. Since the effect was noted even at the lowest specific activities ( $\sim 300$  mCi/mmole) it is not possible to avoid some radiation damage if adequate cpm are to be obtained.

Throughout this study 5 Ci/mmole has been arbitrarily selected for all experiments as it yielded a suitable number of counts. However, from this particular experiment, it is evident that for routine use a lower specific activity would be more appropriate which, although giving a lower incorporation of label, will more accurately reflect the response to PHA.

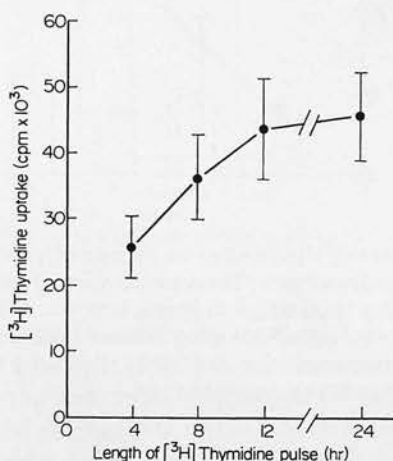


FIG. 5. Effect of pulse time with  $[^3\text{H}]$ thymidine and the level of incorporation of label by PHA-stimulated lymphocytes from five subjects. Each point represents the mean  $\pm$  s.e.m. of five experiments.

##### 5) Effect of length of $[^3\text{H}]$ thymidine pulse

These experiments examined the period over which saturation conditions with thymidine were maintained. The effect of the length of a continuous pulse was investigated using lymphocytes from five donors and shows (Fig. 5) that even with a thymidine concentration of  $4 \times 10^{-4}$  mM, a constant rate of uptake of label was maintained over 24 hr. This suggests that adequate levels of thymidine were available to provide saturating conditions throughout this period, thus minimizing the dilutional effects from endogenous thymidine. In a further series of experiments, using lymphocytes from the same pool of healthy donors but a higher concentration of thymidine, the accumulated uptake achieved by separate hourly pulses over the selected 4-hr labelling period was compared with a continuous pulse over the same period. Fig. 6 shows that the incorporation level of the continuously pulsed cultures was identical with the level reached by accumulating the individual hourly pulses, again showing that an adequate saturating or flooding level was maintained by a single continuous pulse throughout the 4-hr period.

(6) [ $^3\text{H}$ ]thymidine incorporation at different lymphocyte concentrations

Using lymphocytes from twelve donors, a linear incorporation of [ $^3\text{H}$ ]thymidine was found over the range  $0.1 \times 10^6$ – $0.5 \times 10^6$  cells/ml ( $0.2 \times 10^5$ – $1.0 \times 10^5$  cells/culture) with an almost linear relationship continuing up to  $1 \times 10^6$  cells/ml ( $2.0 \times 10^5$  cells/

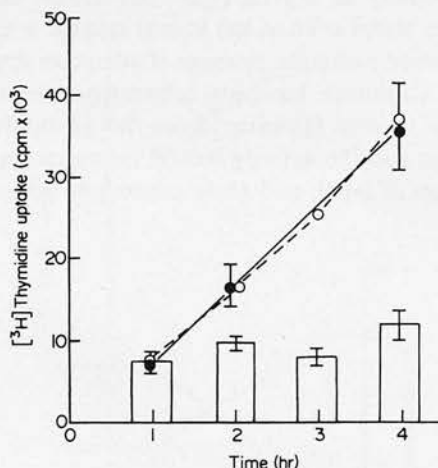


FIG. 6. Effect of exposure time to [ $^3\text{H}$ ]thymidine on the rate of incorporation of label by PHA-stimulated lymphocytes from five subjects. The columns show the mean  $\pm$  s.e.m. of the hourly incorporations, [ $^3\text{H}$ ]thymidine being added at hourly intervals from 44 to 48 hr. (●—●) mean  $\pm$  s.e.m. of continuously pulsed cultures using the same lymphocytes, [ $^3\text{H}$ ]thymidine being added at 44 hr and incubation continuing until 48 hr. (○—○) Cumulative incorporation based on the summation of the hourly incorporations.

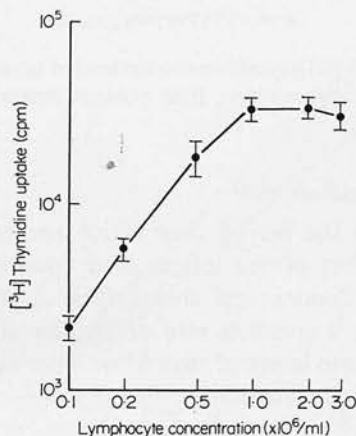


FIG. 7. Influence of lymphocyte concentration on the incorporation of label. Each point represents the mean response  $\pm$  s.e.m. of lymphocytes from twelve donors.

culture) (Fig. 7). Thereafter no further increase in uptake occurred, indicating that an upper limit of  $1 \times 10^6$  cells/ml ( $2.0 \times 10^5$  cells/culture) can be used in this system with optimal numbers in the region of  $0.5 \times 10^6$  cells/ml ( $1 \times 10^5$  cells/culture).

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## (7) Reproducibility of the method

Several aspects of the reproducibility of the method have been examined. In Fig. 8 the variation between individual counts in each triplicate set of a series of a hundred consecutive determinations is expressed as the percentage coefficient of variation. In 93% of the triplicate series this was less than 15%, with the mode between 5 and 10%, indicating that the reproducibility of both culture and extraction procedures was within acceptable limits and similar to the findings of other workers (Schellekens & Eijssvoegel, 1968).

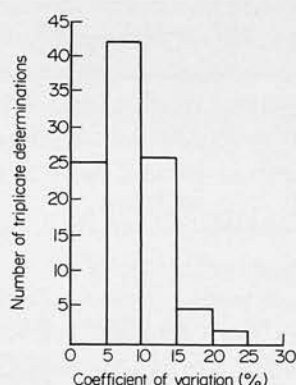


FIG. 8. Percentage coefficients of variation within each triplicate set of a series of 100 consecutive samples.

The reproducibility of the extraction procedure alone was then compared with that of the extensively used method of manifold filtration. The effects of variation due to culture were eliminated by bulking all the cells after [ $^3\text{H}$ ]thymidine labelling and transferring equal aliquots to the filters. Whilst comparable mean cpm were obtained, the coefficient of variation was considerably greater with filtration than with the 'communal rinse' procedure (Table 1).

TABLE 1. Comparison of extraction methods

Method	Number of aliquots counted	Mean cpm $\pm$ s.e.m. ( $\times 10^3$ )	Coefficient of variation (%)
'Communal rinse'	20	69.0 $\pm$ 0.91	6
Filtration	20	59.9 $\pm$ 3.09	23

Finally, the variability observed in repeated samples obtained from a number of healthy persons and patients with organ-specific autoimmune diseases was investigated. In Table 2, day-to-day variations are presented for a group of five normal subjects at one dose of PHA (1.25  $\mu\text{l/ml}$ ) showing the degree of variability to be expected in healthy individuals when fluctuations due to factors such as infection are minimized by frequent sampling. Even under

TABLE 2. Repeated daily PHA response in normal subjects

Subject	Age (years)	Mean cpm ( $\times 10^3$ )			Coefficient of variation (%)
		Day 1	Day 2	Day 3	
Male	31	16.2	15.8	19.1	10.6
Male	29	15.5	14.8	9.5	24.7
Male	34	22.6	33.5	18.3	31.5
Male	32	38.3	21.7	28.6	28.0
Male	26	24.7	21.2	25.9	10.0

TABLE 3. PHA responses over an extended period in normal and autoimmune subjects

Subject	Age/sex	Date	No PHA	PHA ( $\mu$ l/ml)		
				0.3	0.6	1.2
Normal	30/M	23.7.73	0.4	10.5*	25.5	26.1
		20.8.73	0.6	10.9	14.1	14.6
		11.9.73	0.5	8.2	10.5	14.2
		15.10.73	0.6	11.1	15.0	16.4
		4.11.73	0.4	10.5	15.1	17.1
Normal	28/M	23.7.73	0.2	15.7	20.9	13.5
		16.10.73	0.4	13.1	20.4	18.7
		4.11.73	0.5	14.2	22.7	16.4
Normal	27/M	24.7.73	0.2	16.0	22.8	25.1
		20.8.73	0.7	22.1	19.8	21.3
		15.10.73	0.6	18.5	20.4	24.6
Normal	31/M	25.7.73	0.3	21.6	27.4	24.4
		4.11.73	0.6	15.6	24.2	19.9
Normal	32/M	6.8.73	0.5	13.9	14.8	15.2
		4.11.73	0.6	14.1	16.6	20.0
Normal	29/M	2.8.73	0.3	18.3	22.0	26.6
		12.9.73	0.6	3.4	5.4	5.7
Normal	26/F	2.8.73	0.4	14.4	26.0	24.5
		12.9.73	0.5	13.0	21.6	12.0
Normal	24/F	8.10.73	0.3	10.5	16.3	15.8
		31.10.73	0.2	12.9	15.6	14.9
Normal	37/F	4.9.73	0.4	10.0	12.5	16.7
		31.10.73	0.3	10.2	12.0	18.4
Pernicious anaemia	63/F	14.8.73	0.6	8.0	9.0	11.9
		4.9.73	0.9	6.5	7.6	11.7
Pernicious anaemia	74/F	14.8.73	0.5	3.7	6.9	9.1
		4.9.73	0.7	3.2	6.8	13.3
Addison's disease	46/M	21.8.73	0.3	2.6	6.3	4.4
		14.9.73	0.6	2.6	6.3	4.7

\* Cpm  $\times 10^3$ ; mean of triplicate determinations.

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these circumstances it is evident that the coefficient of variation may be as high as 30% between samples from the same subject. The results of repeated sampling of normal subjects and patients with autoimmune disease, over a much wider period of time, are shown in Table 3. On those samples tested more than twice, a mean coefficient of variation of 15% was found over the range of three PHA doses. However, in some individuals, the variation was again in the region of 30%.

Although precise age- and sex-matched controls are not presented here with which to compare autoimmune patients, it can be seen that in these patients the counts are considerably lower than in the normal subjects. This discrepancy cannot be accounted for by the inherent variability between samples. A depression of PHA transformation has been found to be a regular feature of certain forms of organ-specific autoimmunity and is currently under further investigation in this laboratory (MacCuish *et al.*, to be published).

## DISCUSSION

Doubts have recently been expressed concerning the selectivity of PHA for human thymus-derived T lymphocytes, as evidence has been presented showing that in man some B lymphocytes are also stimulated to transform (Phillips & Roitt, 1973). However, the test has proved of value in revealing immunological deviations in a number of clinical states, and its use in this context is likely to continue; it may, perhaps, come to be regarded as a convenient means of assessing the functional level of the recirculating pool of lymphocytes in general, rather than the T-cell element specifically.

Fitzgerald (1971) has shown that in certain immunodeficiency states the dose of PHA which gives the most sensitive discrimination between normal and abnormal response is not necessarily that producing the maximum response. It is probable that the same effect holds for other clinical disorders and it is therefore desirable to use a range of doses of PHA with each lymphocyte sample rather than a single dose. The value of a rapid micro-method requiring small numbers of cells in this context is obvious. Most previous clinical studies of lymphocyte transformation with PHA have employed cell concentrations of  $10^6$  or more per culture. Under the conditions we describe it has been found not only more expedient to use fewer cells but also necessary, since  $10^6$ /ml was found to be the upper limit of linearity when the relationship between lymphocyte numbers and [ $^3$ H]thymidine uptake was investigated. This finding is in agreement with the conclusions of others including Yamamura (1973). Using the micro-method as described, and a standard concentration of  $0.5 \times 10^6$  cells per ml ( $1 \times 10^5$  cells/culture), it is possible to investigate the full dose-response profile to PHA, with all samples in triplicate, using a total of  $2 \times 10^6$  lymphocytes. This number can readily be obtained from 5 ml of peripheral blood.

Earlier studies have also used longer culture periods, usually of 72 hr. Under the present conditions we have found that there is nothing to be gained by culturing for this duration since a higher incorporation of label is attained using the shorter, more convenient time of 3 hr.

A recent investigation of thymidine kinetics in human lymphocyte transformation (Sample & Chretien, 1971) has emphasized the need to obtain optimal labelling conditions; this is necessary to achieve maximum efficiency of labelling. Furthermore, DNA synthesis by different cell populations can only be compared correctly if the same intracellular activity of labelled precursor is reached in each population. These conditions can only be achieved

if the culture is saturated, or flooded, with thymidine throughout the labelling period. It is therefore important to determine the optimal conditions for a particular method, as has been done in the present study, before it is applied to routine use.

When determining optimal labelling conditions it is also necessary to ensure that intranuclear disintegrations resulting from the incorporation of labelled precursor do not cause unacceptable radiation damage to the cells. Drew & Painter (1962) have shown that the extent of cellular damage is a product of the specific activity of the label and the duration of exposure. In the present study we have found evidence that even at the lower specific activities there is an appreciable effect due to internal radiation, and this becomes progressively more severe with increasing specific activity. This effect does not seem to have been taken into account in many clinical studies where isotope of high specific activity is frequently used (up to 30 Ci/mmol in some studies) and may be combined with relatively long labelling periods (up to 3 days). Since it appears that some cell damage due to this effect is unavoidable it is necessary to compromise and use the lowest specific activity conducive with adequate cpm over the shortest possible pulse time. For routine use it is therefore suggested that the specific activity of the isotope should not be greater than 2 Ci/mmol and the pulse time no longer than 4 hr.

The extraction of DNA by drying and 'communal rinsing' has been found to have a number of advantages over more extensively used methods.

(1) It requires less time for the extraction of a given number of cultures than methods based either on centrifugation or filtration.

(2) It is more sparing of reagents.

(3) No specialized or expensive equipment is necessary.

(4) Both counting vials and scintillant can be re-used.

(5) It has proved to be more reproducible in our hands than either centrifugation or filtration procedures.

In conclusion, we recommend the following conditions for the PHA transformation test.  $0.5 \times 10^6$  lymphocytes/ml ( $10^5$ /culture) are cultured in EBM with 10% foetal calf serum for 44 hr in the presence of PHA at concentrations of 0.32, 0.63 and 1.25  $\mu$ l/ml culture. Thereafter [ $^3$ H]thymidine of specific activity 2 Ci/mmol is added at a final concentration of  $8 \times 10^{-4}$  mM, and the cultures incubated for a further 4 hr before termination. Studies of the effect using culture media other than EBM have not been undertaken.

The use of a dose-response curve to PHA, rather than a single concentration, may facilitate the identification of patients with abnormalities of cell-mediated immunological function. This response curve requires to be recalibrated for each batch of PHA.

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# Phytohemagglutinin Transformation and Circulating Lymphocyte Subpopulations in Insulin-dependent Diabetic Patients

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## SUMMARY

The lymphocyte transformation response to the mitogen phytohemagglutinin (PHA) was determined in forty well controlled insulin-dependent diabetics, forty matched normal subjects and fourteen poorly controlled insulin-dependent diabetics. There was no significant difference in the PHA responses of normal subjects and well controlled diabetics, but poorly controlled diabetics showed a marked depression of lymphocyte transformation.

Peripheral blood T and B lymphocyte subpopulations were also measured in fifteen normal subjects, fifteen well controlled diabetics and ten poorly controlled diabetics. The results showed no significant difference between normal and diabetic subjects, whether well or poorly controlled.

The depressed PHA response in poorly controlled diabetics would seem to reflect inadequately corrected metabolic disturbance rather than an inherent, genetically determined immunologic abnormality. *DIABETES 23:708-12, August, 1974.*

Recent studies have examined the role of cell-mediated immune mechanisms in diabetes mellitus. For example, the leucocyte migration test (LMT)<sup>1</sup> has reportedly shown cellular hypersensitivity in diabetics against both nonspecific antigens (human and rat liver mitochondria<sup>2</sup>) and an antigen derived from the microsomal fraction of the islets of Langerhans (porcine pancreatic antigen<sup>3</sup>). In the latter study a positive correlation was found between the LMT response and delayed-type skin hypersensitivity to the antigen.<sup>3</sup>

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Other investigators have used the lymphocyte transformation response to the mitogen phytohemagglutinin (PHA)<sup>4</sup> as an in vitro test of cell-mediated immune function: some record the PHA response to be impaired with diabetic lymphocytes,<sup>5</sup> suggesting immunologic abnormality, while others report it to be normal.<sup>6</sup> To re-examine these results we have measured the lymphocyte transformation response to PHA in well controlled and poorly controlled insulin-dependent diabetics and in normal subjects; in addition erythrocyte rosette<sup>7-9</sup> and in indirect immunofluorescence<sup>7,9</sup> techniques have been used to compare the numbers of circulating T and B lymphocytes in peripheral blood from these three groups.

## PATIENTS AND METHODS

### *Patients*

PHA responses were studied in forty well controlled diabetic and forty normal subjects who were carefully matched for age and sex. The diabetic subjects (twenty-two women, eighteen men; mean age 42.4 years) were insulin-dependent, attended an outpatient clinic, and were free from infection on the day of study. The control subjects were healthy volunteers, mainly laboratory personnel or hospital outpatients not known to have endocrine disease or immunologic abnormality.

PHA responses were also studied in a group of fourteen poorly controlled insulin-dependent diabetics. These patients had been brought to an outpatient clinic for routine or emergency review and their disorder was judged to be poorly controlled by the following criteria: midmorning blood glucose exceeding 350

mg./100 ml.; recent increase in insulin requirements; the presence of heavy glycosuria and/or ketonuria. Some were subsequently admitted to the hospital for correction of their metabolic abnormalities but none had received antibiotics or drugs other than insulin at the time of study. Their clinical details are presented in table 1.

T and B cell subpopulations were measured in fifteen of each of the well controlled diabetic and normal subjects and in ten of the poorly controlled diabetic subjects.

#### Collection of blood samples and separation of lymphocytes

Venous blood was withdrawn at midmorning and was anticoagulated with preservative-free heparin (Weddell Pharmaceuticals or Evans Medical). A portion of each sample was used for measurement of total and differential white blood cell counts. Lymphocytes were separated from the remainder by density centrifugation on a Ficoll-Trisil gradient<sup>10</sup> and washed three times in Eagles' Basal Medium (EBM, Wellcome Reagents Ltd.). The cells were then resuspended in EBM with 10 per cent fetal calf serum (Wellcome), counted and the concentration adjusted to  $1 \times 10^6$  cells per milliliter. All cell suspensions contained more than 95 per cent lymphocytes with a viability greater than 98 per cent on trypan blue exclusion. Lymphocytes from the same sample were used for the PHA test and for T and B cell estimations.

#### Lymphocyte culture with PHA

Stock PHA (PHA-P, Difco) was diluted in EBM

with 10 per cent fetal calf serum to give three solutions containing respectively 0.32, 0.63 and  $1.25 \mu\text{l}$ . PHA per milliliter. Aliquots of  $20 \mu\text{l}$ . of each solution were pipetted into the wells of Cooke Microtiter trays (Flow Laboratories) and  $2 \times 10^5$  cells added to each well. All determinations were performed in triplicate and included control cultures without PHA. The microtrays were gassed with an air-5 per cent  $\text{CO}_2$  mixture and incubated at  $37^\circ \text{C}$ . in sealed containers.

$^3\text{H}$ -thymidine of specific activity 5 Ci./mmol (Radiochemical Centre, Amersham) was diluted with sterile saline to give a working concentration of  $2 \mu\text{Ci./}100 \mu\text{l}$ . Twenty microliters of this solution (i.e.  $0.4 \mu\text{Ci.}^3\text{H}$ -thymidine) was added to each culture after forty-four hours' incubation. The cultures were regassed and reincubated for four hours. The contents of the wells were then pipetted on to fiberglass filter papers (Whatman GF/C) which were air-dried, washed successively with cold 5 per cent trichloroacetic acid, phosphate buffered saline and absolute methanol, and finally placed in Packard glass counting vials. Five milliliters of scintillation fluid (NE233, Nuclear Enterprises Ltd.) was added to each vial and the samples counted for sixty seconds in an automatic beta counter (Packard 2425), the results being expressed as counts per minute (cpm).

#### Identification of T lymphocytes by sheep erythrocyte rosettes

The technic used<sup>7</sup> to identify E-rosettes was derived from that of Jondal et al.<sup>8</sup> and incorporated the modifications of Stjernsward et al.<sup>9</sup>

#### Identification of B lymphocytes

Two technics were used: a rosette technic<sup>8,9</sup> using sheep red cells coated with antibody and complement (EAC rosettes), and indirect immunofluorescence,<sup>7,8</sup> whereby the cells are distinguished by surface immunoglobulin marker.

## RESULTS

#### Lymphocyte transformation with PHA

The dose-response curves of lymphocytes from well controlled diabetic and normal subjects are shown in figure 1. The mean transformation responses in the diabetics to the three doses of PHA employed were  $17.0 \pm 4.3$ ,  $20.8 \pm 4.2$  and  $21.4 \pm 4.3$ , respectively (expressed as  $\text{cpm} \times 10^3 \pm \text{S.E.M.}$ ). The corresponding values in the controls were  $15.3 \pm 4.3$ ,  $19.7 \pm 3.9$  and  $21.8 \pm 3.9$ . Both curves are virtually identical and the lymphocyte transformation response to PHA is not abnormal in well controlled diabetics.

The dose-response curve of lymphocytes from the fourteen poorly controlled diabetics is compared in

TABLE 1  
Clinical details of the fourteen poorly controlled diabetic patients in whom studies were undertaken

Age/ Sex	Duration of Diabetes (yrs.)	Blood glucose (mg. per cent)	Cause of poor control
24/F	10.0	358	Depressive illness
37/F	0.3	355	Recent diagnosis. Establishing control
48/F	5.2	450	Urinary tract infection
60/F	16.0	355	Emotional stress
61/F	11.0	352	Upper respiratory tract infection
66/F	5.0	440	Foot infection
68/F	0.05	385	New diagnosis. Establishing control
69/F	7.1	650	Congestive cardiac failure
17/F	6.9	510	Ketoacidosis, cause unknown
19/M	10.0	365	Skin sepsis, chest infection
28/M	13.0	408	Alcoholic, drinking bout
45/M	6.0	400	Acute bronchitis
46/M	9.0	360	Dental infection
60/M	3.7	800	Bronchopneumonia

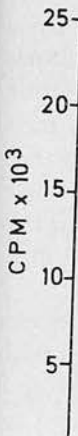


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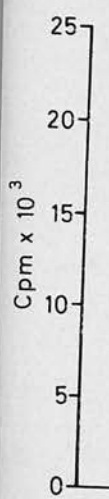


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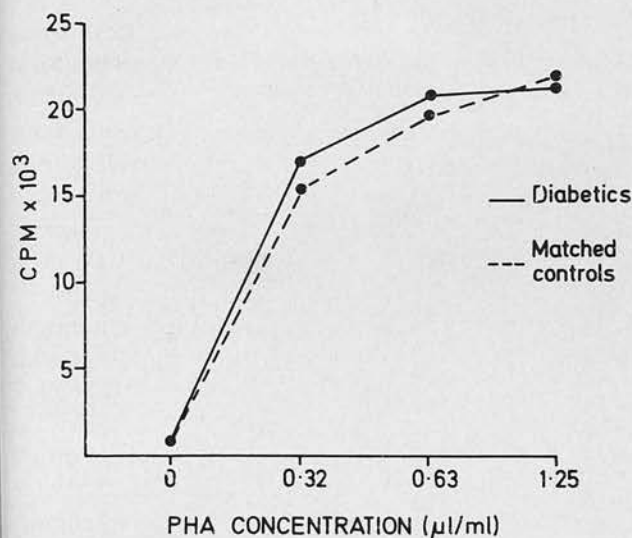


FIG. 1. Mean lymphocyte transformation responses in forty well controlled insulin-dependent diabetic (—) and forty age- and sex-matched normal subjects (----) at three concentrations of PHA. The two dose-response curves do not differ significantly. S.E.M.'s are omitted for clarity but are given in the text.

figure 2 with the curve obtained from fourteen of the well controlled diabetics, matched as closely as possible for age, sex and duration of diabetes. The mean transformation responses in poorly controlled diabetics to the three doses of PHA were  $9.0 \pm 1.33$ ,  $9.5$

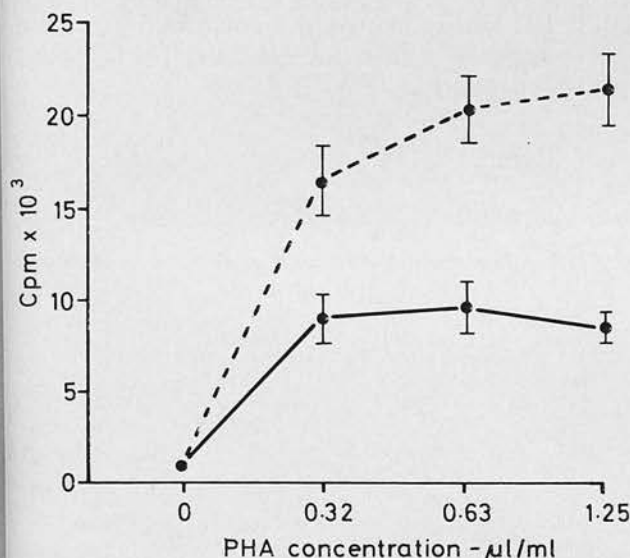


FIG. 2. Lymphocyte transformation responses (mean  $\pm$  S.E.M.) in fourteen poorly controlled insulin-dependent diabetics (—) and fourteen well controlled insulin-dependent diabetics (----), who were matched for age, sex and duration of diabetes. The mean response in the poorly controlled diabetics is significantly lower ( $p < 0.001$ ) at all concentrations of PHA.

$\pm 1.4$  and  $8.2 \pm 0.6$ , respectively. The corresponding values in well controlled diabetics were  $16.5 \pm 2.0$ ,  $20.2 \pm 1.9$  and  $21.3 \pm 2.0$ . These results show a statistically significant reduction of PHA response in poorly controlled insulin-dependent diabetics at all three concentrations of PHA ( $p < 0.001$  by the Student  $t$  test).

#### Populations of T and B lymphocytes in peripheral blood

Tables 2, 3 and 4 list the total lymphocyte counts and the numbers of circulating T and B cells, expressed both as absolutes and percentages of total lymphocyte counts, in samples taken respectively from fifteen well controlled diabetics, fifteen normal subjects and ten poorly controlled diabetics. The results show no significant difference in the mean number and percentage of T cells (assessed by E-rosettes) or B cells (assessed by EAC rosettes or immunofluorescence techniques). Circulating lymphocyte subpopulations are therefore normal in diabetics when obtained at times of 'good' and 'poor' metabolic control.

#### DISCUSSION

The above results confirm that lymphocyte transformation to blast cells by PHA, generally accepted as an *in vitro* test of cell-mediated immunologic response,<sup>11,12</sup> is normal in well controlled insulin-dependent diabetics when compared with matched normal controls. This agrees with the results of Ragab et al.<sup>6</sup> who found no differences in the PHA responses of lymphocytes from twenty-three diabetics and twenty-four controls. Contrary results (depression of PHA response) reported by Brody and Merlie<sup>5</sup> were based on the observations in six elderly diabetics who had persistent glycosuria and marked hyperglycemia (blood glucose 300 to 514 mg. per 100 ml.), and had not taken insulin between twelve and twenty-four hours before the lymphocytes were obtained for study. It therefore appears likely that these results reflect the metabolic disturbance in poorly controlled diabetes, rather than any inherent immunologic abnormality, and the present findings in poorly controlled diabetic subjects agree with this hypothesis.

Further support for the view that depressed PHA response in diabetics is due to metabolic abnormality is provided by the finding of normal numbers of circulating T and B lymphocytes in diabetics, irrespective of whether they were well or poorly controlled at the time of study. Since PHA response is considered mainly to test the function of the T-cell population,<sup>11</sup> it would be surprising to find a depression of PHA response in subjects with normal numbers of T cells;



TABLE 2

Total lymphocyte counts and subpopulations of T and B lymphocytes in peripheral blood from fifteen well controlled insulin-dependent diabetic patients. (E) = T lymphocytes identified by sheep erythrocyte rosettes. (EAC) = B lymphocytes identified by erythrocyte-antibody-complement rosettes. (IF) = B lymphocytes identified by indirect immunofluorescence.

Age/Sex	Total Lymphocyte count/mm <sup>3</sup>	T-cells (E) number	T-cells (E) per cent	B-cells (EAC) number	B-cells (EAC) per cent	B-cells (IF) number	B-cells (IF) per cent
22/F	1,403	878	62.6	449	32.0	—	—
28/F	2,350	1,465	62.3	—	—	357	15.2
35/F	1,566	1,038	66.3	188	12.0	227	14.5
55/F	2,560	1,318	51.5	—	—	294	11.5
56/F	2,211	1,282	58.0	104	4.7	736	33.3
59/F	1,320	854	64.7	121	9.2	300	22.7
62/F	671	374	55.8	203	30.3	221	33.0
62/F	1,360	743	54.6	—	—	299	22.0
68/F	2,132	1,552	72.8	544	25.5	452	21.2
17/M	2,464	1,627	65.9	315	12.8	490	19.9
19/M	3,250	1,570	48.3	504	15.5	523	16.1
36/M	1,975	1,284	65.0	375	19.0	257	13.0
42/M	1,682	1,182	70.3	579	34.4	—	—
63/M	1,440	935	64.9	—	—	2,321	16.1
Mean	1,879	1,153	62.0	338	19.5	375	20.4
± S.E.M.	165	91	1.79	56	3.29	42	1.98

except perhaps in diseases such as breast cancer,<sup>13</sup> multiple sclerosis,<sup>14</sup> or active syphilis<sup>15</sup> where the serum itself may contain factors inhibiting lymphocyte transformation, and in subjects treated with the drug co-trimoxazole.<sup>16</sup> The possibility of finding inhibitory factors in the sera of poorly controlled diabetics is presently being investigated and preliminary results suggest that hyperglycemia per se may contribute to depressed lymphocyte transformation in these patients. Finally, it should be noted that the demonstration of a normal PHA response and T-cell popula-

tion in well controlled insulin-dependent diabetics does not exclude the possibility that smaller numbers of circulating lymphocytes in such patients may show transformation when exposed to a specific antigen, perhaps derived from pancreatic islet tissue.

## ACKNOWLEDGMENT

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TABLE 3

Total lymphocyte counts and subpopulations of T and B lymphocytes in peripheral blood from fifteen normal subjects. Abbreviations as for table 2.

Age/Sex	Total Lymphocyte count/mm <sup>3</sup>	T-cells (E) number	T-cells (E) per cent	B-cells (EAC) number	B-cells (EAC) per cent	B-cells (IF) number	B-cells (IF) per cent
27/F	1,333	857	64.3	216	16.2	—	—
27/F	710	474	66.8	192	27.0	90	12.7
38/F	1,976	1,320	66.8	174	8.8	326	16.5
47/F	1,606	896	55.8	527	32.8	390	24.3
55/F	1,817	950	52.3	136	7.5	—	—
57/F	770	506	65.7	218	28.3	123	16.0
57/F	2,667	1,560	58.5	494	18.5	416	15.6
64/F	2,184	1,223	56.0	109	5.0	618	28.3
69/F	2,054	1,516	73.8	496	24.1	251	12.2
18/M	3,000	2,223	74.1	1,125	37.5	690	23.0
25/M	700	513	73.3	86	12.3	123	17.5
35/M	2,379	1,421	59.3	43	1.8	816	34.3
42/M	1,974	1,137	58.4	516	26.5	405	20.8
66/M	1,672	1,167	69.8	274	16.4	—	—
Mean	1,855	1,194	64.4	323	19.4	415	20.5
± S.E.M.	194	112	1.64	73	4.02	60	2.56



TABLE 4

Total lymphocyte counts and subpopulations of T and B lymphocytes in peripheral blood from ten poorly controlled insulin-dependent diabetic patients. Abbreviations as for table 2.

Age/Sex	Total Lymphocyte count/mm <sup>3</sup>	T-cells (E) number	T-cells (E) per cent	B-cells (IF) number	B-cells (IF) per cent
24/F	1,035	735	71.0	207	20.0
37/F	2,700	2,173	80.5	259	9.6
60/F	1,936	1,073	55.4	407	21.0
61/F	2,430	1,638	67.4	676	27.8
66/F	2,436	1,571	64.5	470	19.3
68/F	1,850	1,166	63.0	487	26.3
19/M	600	303	50.5	102	17.0
28/M	1,472	883	60.0	202	13.7
45/M	1,971	1,242	63.0	242	12.3
46/M	1,950	1,225	62.8	558	28.6
Mean	1,838	1,201	63.8	361	19.5
± S.E.M.	145	109	1.59	38	3.25

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# Autoimmunological Aspects of Diabetes Mellitus

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Until recently, diabetes mellitus has been a disease surprisingly neglected by clinical immunologists but this situation is now being remedied and the past five years in particular have seen an increasing volume of research effort directed to this common endocrinopathy. In this chapter, the current state of knowledge in the following aspects of autoimmunity as related to diabetes mellitus is reviewed:

1. The clinical associations between diabetes and other diseases in which autoimmunity is postulated or established as a major aetiological factor.
2. The serological associations of diabetes with other autoimmune diseases, i.e., studies on the prevalence of serum antibodies to non-pancreatic antigens in diabetics.
3. Studies of serum antibodies to pancreas and pancreatic hormones in diabetes.
4. Studies of cell-mediated autoimmune mechanisms in diabetes.
5. Studies of histocompatibility (HL-A) antigen typing in diabetes.
6. Pathological changes in the pancreas of early or untreated diabetes.
7. Viral infection in relation to the onset of diabetes.
8. Immunological abnormalities in relation to the microvascular complications of diabetes.

The studies reviewed here are mainly concerned with human diabetes mellitus; the most important animal models in which experimental diabetes has been induced by immune mechanisms are also briefly considered.

## CLINICAL ASSOCIATIONS BETWEEN DIABETES AND AUTOIMMUNE DISORDERS

Some of the earliest and most compelling indirect evidence for abnormal immune mechanisms in diabetes has been provided by the clinical observation of an association between diabetes and other disorders of putative or established autoimmune aetiology. Coexisting diabetes and adrenal insufficiency

in a single patient was first recorded in the mid-nineteenth century (Ogle, 1866) and numerous subsequent studies have reinforced the view that the two disorders—one common, one rare—are linked more intimately than can be accounted for by chance (Balfour and Sprague, 1949; Faber and Gronbaek, 1956; Beaven et al, 1959; Solomon et al, 1965; Irvine and Barnes, 1972; Nerup, 1974). From these and other studies the prevalence of diabetes in Addison's disease has been reported as between seven and twenty-three per cent, with an approximate average of eighteen per cent (Irvine and Barnes, 1974), thus representing at least a six-fold excess over the estimated prevalence (one to three per cent) of diabetes in the overall population of Britain or the United States (Marks, Krall and White, 1971). Fewer investigators have attempted to estimate the prevalence of Addison's disease in diabetes but Kozak (1971) recorded a figure of 0.023 per cent among newly-diagnosed cases of diabetes at the Joslin Clinic and Nerup (1974) reported a very similar figure (0.028 per cent) when he examined the prevalence of Addison's disease in a population of fifty thousand Danish diabetics. The prevalence of Addison's disease in the general population has been estimated at between 0.0039 per cent (Stuart-Mason et al, 1968) and 0.0060 per cent (Nerup, 1974b); thus the excess prevalence of Addison's disease in diabetics appears to be in the order of five-fold over the general population.

The development of more sophisticated diagnostic aids, in particular the ability to detect organ-specific humoral antibodies (Anderson et al, 1957; Blizzard and Kyle, 1963), has confirmed that diabetes is usually associated with idiopathic (autoimmune) adrenalitis rather than tuberculous adrenal destruction, and this is borne out by pathological studies. One extensive review of the literature noted idiopathic adrenal atrophy in seventy-four per cent, and tuberculous adrenal involvement in only twenty-two per cent of those patients with dual disease who came to autopsy, and a similar pattern of adrenal pathology was demonstrated in diabetics with combined thyroid and adrenal dysfunction (Solomon et al, 1965). It has also become clear that autoimmune adrenal disease is associated with the juvenile-onset (insulin-dependent) rather than the maturity-onset (insulin-independent) type of diabetes, that adrenal failure precedes pancreatic failure and vice versa in approximately equal numbers, and that both diseases make a simultaneous clinical appearance in the minority of cases (Irvine and Barnes, 1974; see also Chapter 8).

The association between diabetes and pernicious anaemia was first reported in 1910 (Parkinson, 1910) and since then has been described by several investigators (Arapkis et al, 1963; Ungar et al, 1968; Irvine et al, 1970; Munichoodappa and Kozak, 1970). The prevalence of diabetes in patients with pernicious anaemia is said to be seven per cent (Ungar, Whittingham and Francis, 1967), again well in excess of the figure for the general population, and conversely the prevalence of pernicious anaemia in selected diabetic populations has been variously estimated as 0.39 per cent (Chanarin, 1964), four per cent (Ungar et al, 1968) and five per cent (Irvine et al, 1970). The lower figures probably underestimate the true position as pernicious anaemia is often of latent form in diabetics and associated with intrinsic-factor antibodies (frequently suggested as the 'marker' of latent or frank pernicious



anaemia) in only fifty to sixty per cent of cases (Ardeman and Chanarin, 1963; Irvine, 1965). An accurate estimate of prevalence is therefore only achieved when the clinician is prepared to screen all diabetics at risk (mainly middle-aged to elderly females, irrespective of antidiabetic treatment) not only for intrinsic-factor antibodies but also for an adequate serum vitamin B<sub>12</sub> level (Ungar, Whittingham and Francis, 1967).

Multiple reports have suggested an association between diabetes and thyroid disorders. The diseases in question comprise thyrotoxicosis, usually in juvenile diabetics (Hayles et al, 1959; Perlman, 1961), chronic or Hashimoto thyroiditis in both children and middle-aged patients (Landing et al, 1963; Masi et al, 1965; Crome, Erdohezi and Rivers, 1967), and primary hypothyroidism (Solomon et al, 1965; Hecht and Gerschberg, 1968; Andreani, 1974). Both pernicious anaemia and thyroid disorders occur with significant frequency (three and nine per cent respectively) in the first-degree relatives of diabetic patients (Irvine et al, 1970).

Finally, an association of diabetes with myasthenia gravis has also been recently documented (Osserman, 1969).

### SERUM ANTIBODIES TO NON-PANCREATIC ANTIGENS IN DIABETES MELLITUS

The clinical associations of diabetes with autoimmune diseases are impressive but perhaps even more striking is the high prevalence of certain organ-specific autoantibodies in diabetic serum. Numerous serological studies have been performed in diabetics, directed in particular to the detection of thyroid or gastric autoantibodies, and the results of some of the more extensive investigations are presented in Table 1. These studies have been performed in young and elderly as well as in unselected (random) populations. Patients with overt thyroid or gastric disease have been excluded, and in most instances the results have been compared with those from large non-diabetic populations of comparable age and sex distribution. The overall prevalence of various autoantibodies in diabetics can therefore be calculated with fair accuracy from the pooled data of the major series (Table 2) and more detailed information is given in the following explanatory notes:

#### Thyroglobulin autoantibodies

Most investigators agree that the prevalence of antibodies to thyroglobulin in diabetes (measured by the tanned cell haemagglutination technique) is marginally but hardly significantly increased over that in the non-diabetic population. In both groups the overall prevalence seems to lie between seven and ten per cent (Tables 1 and 2), and major deviations from this relatively narrow range are found only in restricted population strata: for example, the high figures recorded by Maret and Berthaux (1965) in diabetics and controls were obtained from groups whose members were almost exclusively female and all aged over seventy years. Only one study (Simkins, 1968) has described

a major difference between diabetics and controls (prevalence ten and four per cent respectively) and the results obtained by this investigator may be partly explicable by the racial differences between the groups; Simkins examined mixed Caucasian/Negro populations but half the diabetic and only twenty-nine per cent of the control group were negroes.

**Table 1.** *Prevalence of autoantibodies in sera from diabetic and non-diabetic (control) populations, reported by various authors*

Reference	Subjects (No. studied)	Number (per cent) positive for:					
		T/G	T/C	PCA	IF	ANF	ADR
Pettit, Landing and Guest (1961) <sup>a</sup>	Diabetics (58)	—	13 (22)	—	—	—	—
	Controls (229)	—	2 (1)	—	—	—	—
Landing et al (1963)	Diabetics (225)	—	40 (18)	—	—	—	—
Moore and Neilson (1963)	Diabetics (65)	6 (9)	—	—	—	—	—
	Controls (65)	5 (8)	—	—	—	—	—
	Diabetics (83)	—	14 (17)	18 (22)	—	—	—
	Controls (166)	—	7 (4)	13 (8)	—	—	—
Maret and Berthaux (1965) <sup>b</sup>	Diabetics (82)	27 (33)	—	—	—	—	—
	Controls (110)	28 (26)	—	—	—	—	—
Ungar et al (1968)	Diabetics (400)	—	—	84 (21)	8 (2)	—	—
	Controls (600)	—	—	65 (11)	—	—	—
	Controls (1600)	—	—	—	5 (<1)	—	—
Simkins (1968)	Diabetics (317)	32 (10)	—	—	—	—	—
	Controls (424)	17 (4)	—	—	—	—	—
Irvine et al (1970)	Diabetic (1054)	90 (9)	170 (16)	176 (17)	—	11 (1)	—
	Controls (871)	56 (6)	68 (8)	53 (6)	—	9 (1)	—
	Diabetic (380)	—	—	—	13 (3)	—	—
	Controls (296)	—	—	—	1 (<1)	—	—
Goldstein et al (1970) <sup>c</sup>	Diabetics (252)	22 (9)	—	15 (6)	—	—	0 (0)
	Controls (340)	20 (6)	—	4 (1)	—	—	0 (0)
Whittingham et al (1971)	Diabetics (400)	—	56 (14)	84 (21)	8 (2)	88 (22)	—
	Controls (400)	—	34 (9)	40 (10)	0 (0)	110 (27)	—
Nerup and Binder (1973)	Diabetic (133)	13 (10)	27 (20)	21 (16)	—	3 (2)	3 (2)
	Controls (128)	10 (8)	4 (3)	7 (5)	—	0 (0)	0 (0)
MacCuish et al (1974a)	Diabetics (250)	23 (9)	43 (17)	45 (18)	—	3 (1)	—
	Controls (250)	18 (7)	21 (8)	20 (8)	—	2 (1)	—

<sup>a</sup>All subjects aged under 16 years.

<sup>b</sup>All subjects aged over 70 years.

<sup>c</sup>No distinction between antibodies to thyroglobulin and thyroid cytoplasm.

T/G = antibody to thyroglobulin.

T/C = antibody to thyroid cytoplasm.

PCA = antibody to gastric parietal cytoplasm.

IF = antibody to gastric intrinsic factor.

ANF = antinuclear antibody.

ADR = antibody to adrenal cortex.

**Table 2.** Overall prevalence of autoantibodies in sera from diabetic and non-diabetic populations, calculated from the pooled data in Table 1

Subjects (No. studies)	Number (per cent) positive for:					
	T/G	T/C	PCA	IF	ANF	ADR
Diabetic (1901)	191 (10)	—	—	—	—	—
Controls (1848)	134 (7)	—	—	—	—	—
Diabetic (2203)	—	363 (16)	—	—	—	—
Controls (2044)	—	136 (7)	—	—	—	—
Diabetic (2572)	—	—	445 (17)	—	—	—
Controls (2755)	—	—	202 (7)	—	—	—
Diabetic (1180)	—	—	—	29 (2)	—	—
Controls (2296)	—	—	—	6 (< 1)	—	—
Diabetic (1837)	—	—	—	—	105 (6)	—
Controls (1649)	—	—	—	—	121 (7)	—
Diabetic (385)	—	—	—	—	—	3 (1)
Controls (468)	—	—	—	—	—	0 (0)

Abbreviations as Table 1.

### Autoantibodies to thyroid and gastric parietal cytoplasm

Antibodies to thyroid or gastric parietal cell (detected initially by complement fixation, latterly by the Coombs indirect immunofluorescence technique) are conveniently considered jointly as their distribution is very similar in diabetic populations and indeed they may coexist in the same patient. Most investigators (Table 1; Figure 1) record a two- to fourfold increase in diabetics over the expected prevalence of either antibody in the general population, but in selected groups, for example in juveniles, the difference may be even greater (Pettit, Landing and Guest, 1961).

The combined effects of age, sex and antidiabetic therapy on antibody prevalence have been carefully analysed in several studies and allow the following conclusions to be drawn:

1. Thyrogastric antibodies are found most commonly in young female, insulin-dependent diabetics.
2. The prevalence of thyrogastric antibodies in older diabetics (i.e., above age forty) is much closer to that in control populations. Again the excess is mainly associated with female sex and insulin dependency.
3. The joint occurrence of thyroid and gastric antibodies is almost exclusive to diabetics.
4. A high titre of thyroid cell antibodies in ostensibly euthyroid diabetic children may be associated with chronic (Hashimoto) thyroiditis.

The marked difference in antibody prevalence between insulin-dependent and insulin-independent patients is probably accounted for by differing modes of inheritance for juvenile-onset and maturity-onset diabetes. The genetics of diabetes are complex and the modern view favours a multifactorial hypothesis, involving the additive effects of several genes (Falconer, 1967). By this hypothesis overt diabetes results from an amalgam of genetic and environmental factors, the former being strongest in juvenile-onset and the latter in adult-onset diabetes. In this context it is interesting to note that

evidence for genetic heterogeneity in diabetes has been sought, and found, by serological study of the relatives of juvenile diabetics: a significantly higher frequency of thyroid and gastric antibody titres was found among parents and siblings of juvenile diabetics with positive titres than among relatives of juvenile diabetics with negative titres (Nissley et al, 1973).

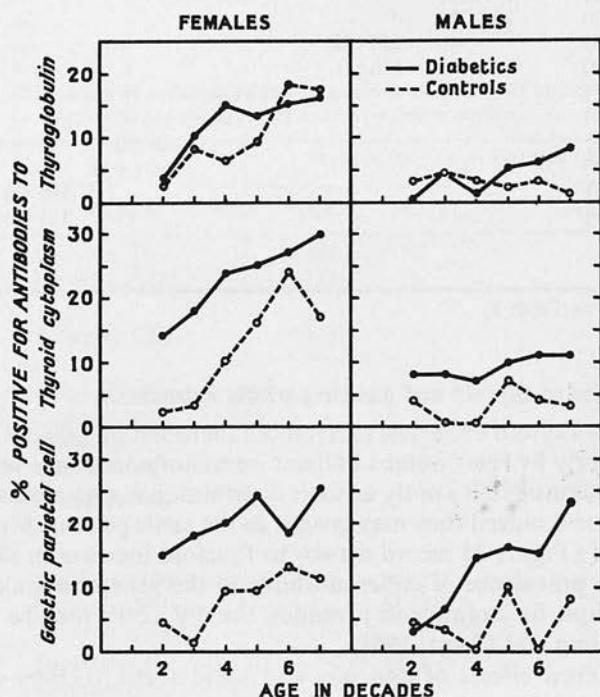


Figure 1. The percentage incidence of autoantibodies specific for thyroglobulin, thyroid cytoplasm and gastric parietal-cell cytoplasm in a series of 1032 diabetics and of 871 controls according to sex and age in decades. Reproduced from Irvine et al (1970) by courtesy of the Editor of *The Lancet*.

The few studies which have related antibody prevalence to duration of diabetes are of particular interest. Some workers have described increasing prevalence of thyrogastric antibodies with lengthening duration of disease, but this relationship only appears to be valid for insulin-taking patients, aged under thirty years, who have been diabetic for more than ten years (Nerup and Binder, 1973). Conversely the most extensive published series found no clearcut relationship between antibody prevalence and duration of disease (Irvine et al, 1970). Whittingham et al (1971) applied a sophisticated computer programme to carefully-matched populations and detected an actual decrease in thyrogastric antibody prevalence with increasing duration of insulin-dependent diabetes, the decrease being especially marked in diabetics of more than twenty years' standing. These authors offered the explanation that their findings reflected an unexpectedly high death rate



among longstanding diabetics with thyrogastric antibodies, and further demonstrated that antibody prevalence in long-term insulin-dependent diabetics aged under forty is equal to that in the non-diabetic population aged over sixty. Serologically, therefore, there is some evidence that the long-term insulin-dependent diabetic population, especially female, has aged prematurely by at least twenty years, but how this phenomenon may contribute to an excess mortality amongst these patients is not yet clear. Apart from degenerative disease of the large blood vessels, there is no other convincing biological evidence of premature aging in diabetes mellitus.

The appearance of serious and so-called specific diabetic complications (renal, retinal, neuropathic, etc.) is of course notoriously associated with insulin-dependent diabetes of at least ten years' duration, and it is to be hoped that future serological studies will explore the relationships between duration and therapy of diabetes, antibody status and appearance of microangiopathy. At present it may be reasonable to accept that the presence of thyrogastric antibodies in young insulin-dependent patients might identify a particular risk factor for early death, and to keep such patients under close surveillance for the development not only of overt thyrogastric disease but also of specific diabetic vascular lesions.

#### **Autoantibodies to gastric intrinsic factor**

Antibodies to intrinsic factor (IF), detected by a radioimmunoassay method using coated charcoal, are found much less commonly than parietal-cell antibodies in diabetes. Nonetheless their presence is of especial importance because of the strong association, mentioned earlier, with underlying pernicious anaemia. Thus Ungar et al (1968) found IF antibody in eight of 400 diabetics studied, three of whom had latent pernicious anaemia, while Irvine et al (1970) found IF antibody in 13 of 380 patients studied, latent pernicious anaemia being present in six of the nine patients who underwent additional investigations. The apparently low prevalence of IF antibody in the overall diabetic population (Table 2) appears in correct perspective when it is appreciated that all investigators who have searched for this antibody are unanimous in indicating that IF antibodies are found only in middle-aged to elderly diabetics, almost exclusively female and predominantly insulin-dependent. In these selected population strata it has been estimated that the prevalence of IF antibody is between four and five per cent (Ungar et al, 1968; Irvine et al, 1970) and half the patients thus identified will have latent pernicious anaemia. These figures represent an increase of at least four-fold over the expected prevalence of IF antibody in matched non-diabetic populations and are sufficiently impressive to reinforce the value of screening for IF antibody in the diabetic population at particular risk.

#### **Autoantibodies to adrenocortical cells**

Few investigators have examined diabetic populations for adrenal antibodies (Table 1). However, it is worth noting that this antibody, which is detectable in more than fifty per cent of patients with idiopathic Addison's disease and is extremely rare outside that context, was found by Nerup and Binder (1973) in three insulin-dependent diabetics with no overt evidence of adrenal

failure. The strong clinical associations of diabetes with Addison's disease have already been commented upon and cell-mediated immunity against adrenal antigen has also been demonstrated in diabetics (Nerup and Bendixen, 1969).

### **Other autoantibodies and antigens in diabetes**

The prevalence of antibodies which are tissue-specific rather than organ-specific, for example mitochondrial antibody and antinuclear factor (ANF), does not appear to be increased in diabetic populations (Table 2). Likewise the prevalence of lymphocytotoxic antibodies is not different in diabetics, whether insulin-dependent or insulin-independent, from that in the normal population (Singal and Blachman, 1973).

## **SERUM ANTIBODIES TO PANCREAS AND PANCREATIC HORMONES IN DIABETES MELLITUS**

In contrast to the striking and widely-documented prevalence of organ-specific antibodies to other tissues, intensive efforts to demonstrate anti-pancreatic or anti-insulin antibodies in diabetes have until recently been largely unrewarding. Early investigators, using haemagglutination, gel-diffusion and precipitin techniques claimed to find antipancreatic antibodies in the sera of a high proportion of the diabetics that they tested, as well as in other forms of chronic pancreatic disease (Murray and Thal, 1960; Fonkalsrud and Longmire, 1961). These results were not confirmed subsequently and were probably due to methodological artefacts (Villavicencio, Thurnau and Goetz, 1965). Efforts to detect antipancreatic antibody by the immunofluorescence technique proved equally fruitless (Irvine et al, 1970; Nerup and Binder, 1973; Doniach, 1974a) and it was assumed that such an immunoglobulin did not exist. Very recently, however, two independent studies have confirmed the existence of an antibody directed against the endocrine pancreas in diabetic sera (Bottazzo, Florin-Christensen and Doniach, 1974; MacCuish et al, 1974a), and the combined results of these studies allow the following conclusions to be drawn:

1. Islet-cell antibody is only detectable in patients with overt autoimmunity and is found almost exclusively in insulin-dependent diabetics in whom diabetes coexists with single or polyglandular autoimmune disease (Table 3). In this selected group of diabetics, the antibody has thus far been found in approximately thirty per cent of sera tested. The simple explanation for the previous failure to detect islet-cell antibody is that the earlier searches examined sera from unselected patients (e.g., insulin-independent diabetics) or deliberately excluded diabetics with overt autoimmunity.

2. Islet-cell antibody is demonstrable in unfixed pancreatic sections by immunofluorescence, is an immunoglobulin of IgG class and fixes complement. It is thus very similar to its established counterparts in autoimmune disease. Fluorescence is abolished by pretreatment of the pancreatic tissue substrate with alcohols, gluteraldehyde and formaldehyde but is unaffected by acetone fixation. These fixative reactions support the belief that the

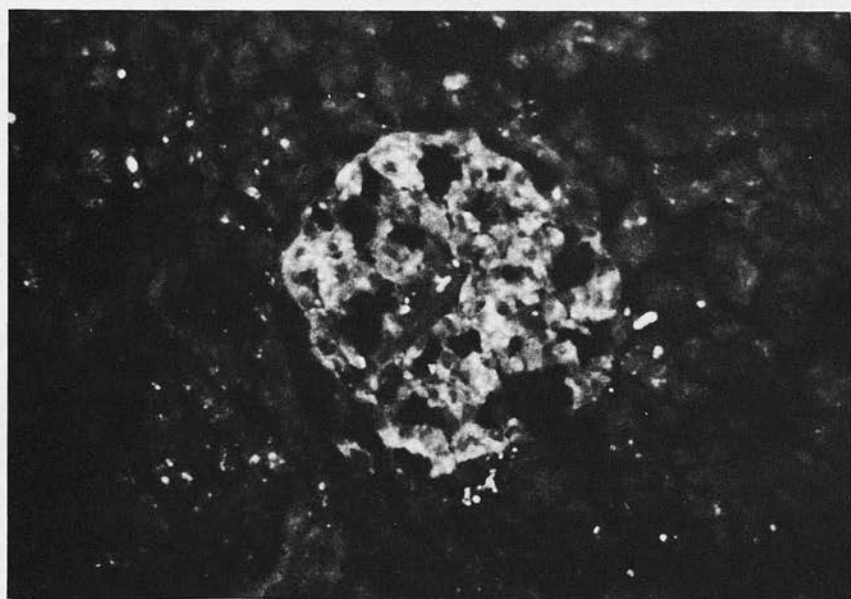


Figure 2. Pancreatic islet-cell antibody in diabetes mellitus. Cryostat section of human pancreas treated with serum of a diabetic patient, followed by anti-human IgG-FITC conjugate, showing cytoplasmic fluorescence over the islet of Langerhans. Fluorescence is apparent in all cell types in the islet.

In the light of present knowledge and techniques, the presence of insulin antibodies should not be accepted unless the serum under study can be proved to contain immunoglobulins, which bind isotope-labelled insulin, by a specific and sensitive method (gel filtration, paper chromatography or dextran-coated charcoal). It is therefore of particular interest to record that by using these elegant techniques, eight cases from Japan (Ohneda et al, 1974) and one from Norway (Følling and Norman, 1972) have been reported of patients who by all reasonable criteria had never taken exogenous insulin and whose serum nonetheless contained insulin-binding (auto)antibodies. The term 'insulin autoimmune syndrome' has been applied to describe the salient clinical and biochemical abnormalities (Hirata et al, 1972); most patients have been studied in considerable detail and have the following features in common:

1. No history of immunisation with insulin.
2. Presentation with post-prandial hypoglycaemic attacks. Demonstration of carbohydrate intolerance (diabetic type) on glucose loading, followed by reactive hypoglycaemia. No fasting hyper- or hypoglycaemia.
3. High titre of insulin-binding antibodies in plasma, the antibodies binding mammalian insulins with ease but having the strongest affinity for human and porcine insulin.
4. Characterisation of the insulin-binding antibody as IgG immunoglobulin of K type (in contrast to injected insulins, which evoke a heterogeneous response of IgG, IgM and IgA antibodies of both K and L type).

pancreatic islet antigen-antibody system will share the other properties common to the thyrogastric autoimmune systems (Doniach, 1974b), and indicate that the antigen is likely to be microsomal, i.e., comprised of membrane lipoproteins from subcellular fractions of smooth endoplasmic reticulum. Absorption and subcellular fractionation studies will however be necessary to characterise the antigen.

**Table 3.** *Prevalence of autoantibodies (IgG class) against pancreatic islet cells, in sera from diabetics and non-diabetics*

Subjects tested	Islet-cell antibody	
	Positive	Negative
Diabetic with co-existent autoimmune disease	15	35
Non-diabetic with clinical autoimmune disease	3	81
Diabetic with positive thyrogastric autoantibodies	0	20
Diabetic with negative autoantibodies	0	64
Non-diabetic with negative autoantibodies	0	58
Total	18	258

Autoantibodies (IgG class) against pancreatic islet cells, detected by indirect immunofluorescence.

Combined results of Bottazo, Florin-Christensen and Doniach (1974) and MacCuish et al (1974a).

3. Islet-cell antibody produces a uniform cytoplasmic immunofluorescence affecting all cell types ( $\alpha$ ,  $\beta$  and  $\delta$ ) in the islets of Langerhans (Figure 2). The exocrine pancreas is unaffected.

The detection of islet-cell antibody in diabetics has two immediate implications: first, it adds strong credence to the concept of an autoimmune form of diabetes, especially when considered in conjunction with the evidence, discussed below, of T-cell sensitisation to pancreatic antigens in diabetes; secondly, the antibody may prove a convenient rapid 'marker' for the autoimmune form of diabetes, the patients thus identified being examined for other evidence of autoimmunity by sophisticated immunological techniques and possibly also for histocompatibility type. It will be particularly important to undertake studies of cell-mediated immune function in diabetics who are shown to possess this humoral antibody.

The situation with respect to anti-insulin antibodies in diabetes is broadly similar. A few workers apparently demonstrated such antibodies, in untreated diabetics, by the complement consumption test (Pav, Jezkova and Skrha, 1963; Chetty and Watson, 1965), the precipitin test (Penchev, Andreev and Ditzov, 1968), or the histoimmunologic technique (Mancini et al, 1965). These procedures are however disputed (Van de Wiel and Van de Wiel-Dorfmyer, 1964; Chao, Karam and Grodsky, 1965) and do not seem specific for insulin antibodies. In contrast Berson and Yalow never observed insulin-binding antibodies in thousands of samples from untreated diabetics, and conversely could readily detect them in the serum of virtually every patient on insulin therapy (Berson et al, 1956; Berson and Yalow, 1965).



1971), and the application of such techniques to the study of human disease, have presented some of the most promising opportunities for demonstrating immunological abnormality in diabetes mellitus. Thus far three main areas of cell-mediated immunity in diabetes have been explored and the results obtained are outlined below.

### **General cellular immune response and circulating lymphocyte subpopulations**

**RESPONSE TO NONSPECIFIC MITOGENS.** The mitogen phytohaemagglutinin (PHA) induces transformation of cultured small lymphocytes to blast cells (Nowell, 1960). Under appropriate conditions, sixty to seventy per cent of lymphocytes are transformed *in vitro* by PHA and the majority of these cells will be T lymphocytes (Roitt *et al*, 1969), although some B lymphocytes are also stimulated to transform (Phillips and Roitt, 1974). PHA testing is therefore extensively employed as a general assessment of cell-mediated immune function in a variety of clinical states and may be regarded as a convenient means of judging the functional level of the recirculating pool of lymphocytes.

Conflicting results have been reported by the investigators who initially performed PHA testing in diabetes. Brody and Merlie (1970) found marked depression of blast transformation in diabetes, comparable to that in patients with chronic lymphatic leukaemia, while Ragab, Hazlett and Cowan (1972) found no difference in the PHA responses of lymphocytes from diabetics and healthy controls. However, it seems clear that these opposing views can be reconciled by relating the PHA response in diabetics to the adequacy of metabolic control when the cells were removed for testing: the report of Brody and Merlie was based on observations in six elderly patients who had persistent glycosuria and hyperglycaemia (blood glucose 300 to 514 mg%) and had not taken insulin for between twelve and twenty-four hours before the lymphocytes were removed for study. In contrast the normal results reported by Ragab *et al* were obtained from twenty-three healthy diabetic outpatients. In our own experience (MacCuish *et al*, 1974b) using a sensitive micromethod assay system (Penhale *et al*, 1974), we have found no difference in the PHA responses of a large number of well-controlled diabetics when compared with a matched healthy population (Figure 3). Conversely we have demonstrated profound depression of lymphocyte transformation in poorly controlled patients (Figure 4). Thus it is reasonable to regard a depressed PHA response as one reflection of inadequately corrected metabolic disturbance in poorly controlled diabetes rather than inherent genetically determined immunological abnormality in this disease.

**T AND B LYMPHOCYTE SUBPOPULATIONS.** Further support for the view that general cellular immune status is normal in well-controlled diabetes has been provided by the demonstration that circulating lymphocyte subpopulations of T and B cells are normal in diabetics, irrespective of the patient's metabolic control and therefore of the PHA response (MacCuish *et al*, 1974b). These findings again point to functional rather than structural lymphocyte abnormality in poorly controlled diabetes, and our own investigations in these patients suggest that both hyperglycaemia and elevated levels of plasma free fatty acids make important contributions to depressing lymphocyte blasto-

5. Hyperinsulinism.
6. Hypertrophy of the islets of Langerhans (in those patients who underwent laparotomy).

The apparently paradoxical response to glucose loading found in the insulin autoimmune syndrome, which in some respects resembles the carbohydrate intolerance of early diabetes, may be explicable on the following basis: insulin secreted in response to glucose is avidly bound by the circulating antibodies (causing hyperglycaemia); blood glucose remains high when the binding capacity is saturated and insulin production continues unchecked from the maximally-stimulated beta cells (causing hypoglycaemia). Small amounts of free insulin, in equilibrium with the large bound fraction, prevent the development of ketoacidosis or fasting hyperglycaemia. The remarkable homogeneity of the antibody identified (IgG in all cases) suggests a monoclonal production.

It is likely that awareness will now lead to further cases of this interesting syndrome being identified. However, the relevance of these findings to the pathophysiology of human diabetes is uncertain; at present it must be concluded that such cases are rare in clinical presentation, that insulin autoantibodies are exceptional, and that the majority of untreated juvenile-onset diabetics do not possess such antibodies. It is probable that there is at best a tenuous connection between the insulin autoimmune syndrome and the immune phenomena described in 'classical' diabetes mellitus.

Antibodies to glucagon, the other major hormone secretion of the islets of Langerhans, have also been recently described in insulin-taking diabetics (Stahl et al, 1972; Cresco et al, 1974). Their appearance is readily explicable on the basis of immunisation by the small quantities of glucagon with which commercial insulins are contaminated. There is no suggestion that they exist in the untreated diabetic. Similar considerations apply to proinsulin antibodies.

Finally, the postulate that diabetes may result from the production of an immunologically or biologically abnormal insulin, by an inflamed and failing pancreas, remains unproven. Studies in diabetics have shown no evidence of structural abnormality of pancreatic insulin (Kimmel and Pollock, 1967), the secretion of the precursor proinsulin into the circulation is unimpaired (Goldsmith, Yalow and Berson, 1969), and the conversion of proinsulin to insulin seems to proceed normally (Rubenstein and Steiner, 1971). However, it has been calculated on theoretical grounds that at least thirty variants of proinsulin could exist in the human population (Steiner, 1972). There is some evidence that plasma insulin in juvenile diabetics is less active biologically than immunologically (Roy, Shapcott and O'Brien, 1968) and it would be well to keep an open mind to the possibility that abnormalities of insulin biosynthesis or structure may eventually be demonstrated in diabetes.

### STUDIES OF CELL-MEDIATED AUTOIMMUNE MECHANISMS IN DIABETES MELLITUS

The major components of the cellular limb of the immune system are summarised elsewhere in this volume (page 289). The recent development of sensitive *in vitro* techniques to investigate cellular immune responses (Bloom,

in diabetes, especially as the other known defects in diabetic defence mechanisms (abnormal chemotaxis and phagocytosis) are probably independent of the metabolic state (Baciu, Derenenco and Vitebski, 1967; Hill et al, 1974).

Finally, it is worth noting that the demonstration of a normal PHA response and T-cell population in well-controlled diabetes has not excluded the possibility that smaller numbers of circulating lymphocytes in such patients may show recognition when exposed to a specific antigen, derived from pancreatic tissue or hormone, as the studies described below have demonstrated.

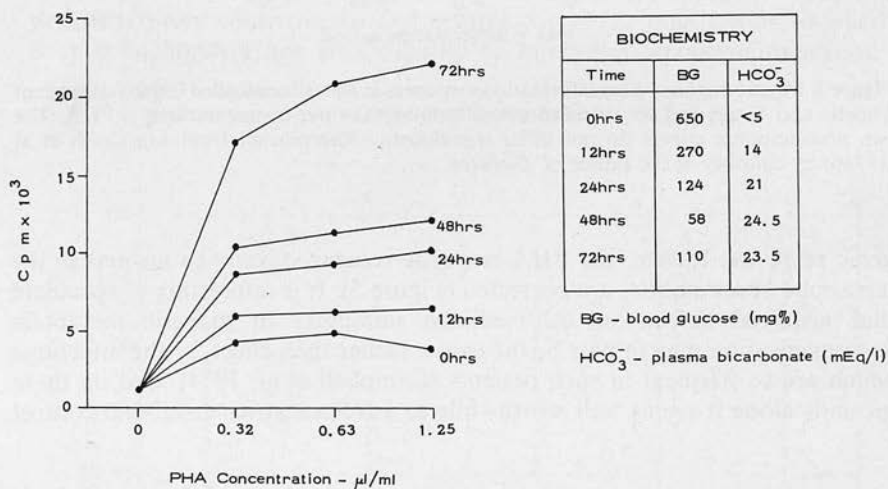


Figure 5. Lymphocyte transformation responses to three concentrations of PHA in an insulin-dependent diabetic during recovery from ketoacidosis. Transformation response is grossly depressed in severe metabolic decompensation but returns to normal within 72 hours following correction of the ketoacidosis.

### Migration inhibition studies in diabetes mellitus

The leucocyte migration technique (LMT), as developed by Bendixen and Soborg (1969) for studies of immune phenomena in human disease, has been used by several investigators to provide evidence of cellular (delayed type) hypersensitivity to specific antigens in diabetes mellitus. The theoretical basis of the test depends on the ability of lymphocytes from a sensitised individual, on contact with specific antigen, to produce a soluble factor(s) which modifies the normal migration of white blood cells in culture medium (Figure 6). The first successful demonstration of antipancreatic cellular hypersensitivity in diabetes by this technique was accomplished by Nerup et al (1971), who prepared an antigen of very high islet-cell content from porcine pancreas in which atrophy of exocrine tissue had been induced by surgical ligation of the

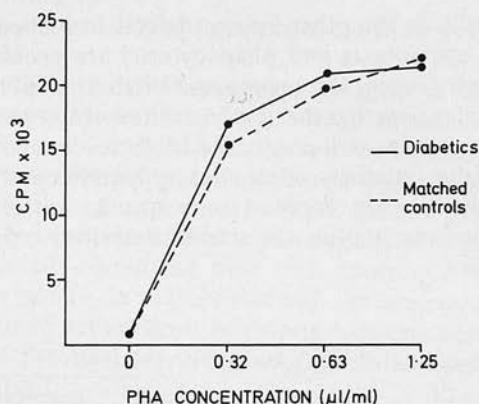


Figure 3. Mean lymphocyte transformation responses in 40 well controlled insulin-dependent diabetic and 40 age and sex-matched normal subjects at three concentrations of PHA. The two dose-response curves do not differ significantly. Reproduced from MacCuish et al (1974b) by courtesy of the Editor of *Diabetes*.

genic response. Indeed, the PHA response returns steadily to normal as the metabolic abnormalities are corrected (Figure 5). It is interesting to speculate that acquired defects of cell-mediated immunity in diabetic metabolic decompensation may in part be the cause, rather than effect, of the infections which are so frequent in such patients (Campbell et al, 1974), and on these grounds alone it seems well worthwhile to advocate strict metabolic control

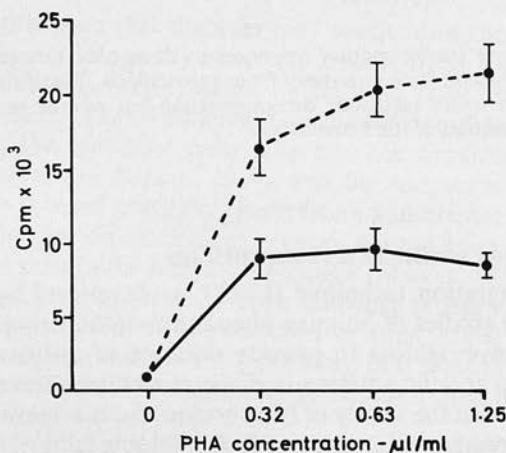


Figure 4. Lymphocyte transformation responses (mean  $\pm$  s.e.m.) in 14 poorly controlled insulin-dependent diabetics (—) and 14 well controlled insulin-dependent diabetics (---) who were matched for age, sex and duration of diabetes. The mean response in the poorly controlled diabetics is significantly lower ( $P < 0.01$ ) at all concentrations of PHA. Reproduced from MacCuish et al (1974b) by courtesy of the Editor of *Diabetes*.



found in diabetics who have never received insulin injections, and in whom the question of prior sensitisation by exogenous antigen does not therefore arise, while no correlation has been found between the presence of insulin antibodies and a positive LMT in treated patients (Nerup et al, 1971, 1973a). Thus it seems fair to regard the studies cited above as a demonstration of a true autoimmune phenomenon in diabetes. The failure of insulin itself to act as antigen in the LMT may merely reflect the technical limitations of this particular test, which yields satisfactory results with particulate but rarely with soluble antigens; alternatively the active antigen may be an insulin precursor (proinsulin, C-peptide) or some other pancreatic protein or cellular constituent.

The position regarding migration inhibition with antigens from other organs is more controversial and centres round the question as to whether liver mitochondria are also capable of inhibiting leucocyte migration in

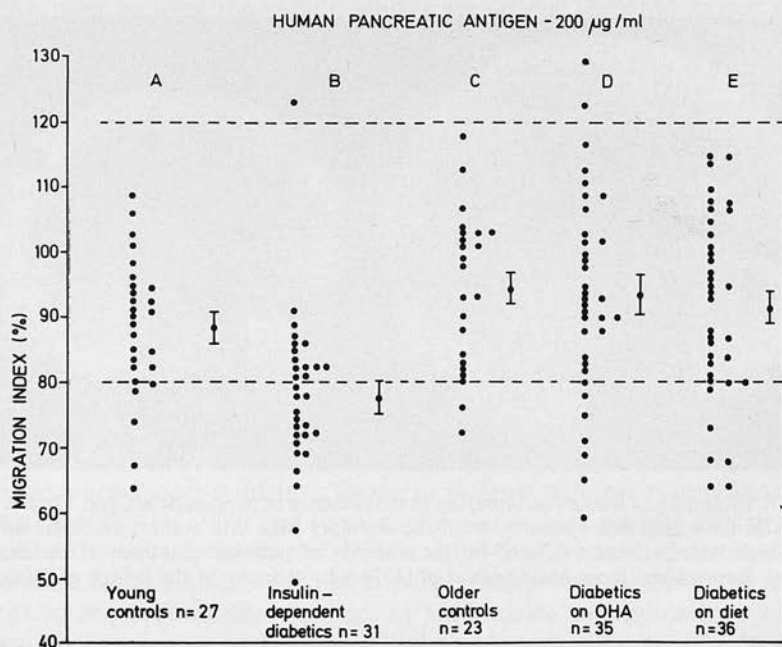


Figure 7. Leucocyte migration test with human pancreas extract in 101 diabetics and 50 controls. Data from MacCuish et al (1974c).

A = 27 healthy young controls (mean age 28.6 years).

B = 31 juvenile-onset diabetics treated with insulin (mean age 28.5 years).

C = 23 healthy older controls (mean age 57.4 years).

D = 35 maturity-onset diabetics on oral hypoglycaemics (mean age 59.7 years).

E = 36 maturity-onset diabetics on diet alone (mean age 60.5 years).

*Wilcoxon's tests*

B-A  $P < 0.01$

B-C  $P < 0.01$

B-D  $P < 0.01$

B-E  $P < 0.01$

A-C  $P > 0.10$

A-D  $P > 0.10$

A-E  $P > 0.10$

C-D  $P > 0.10$

C-E  $P > 0.10$

pancreatic duct for eight weeks before the organ was removed. This antigen induced inhibition of leucocyte migration in peripheral-blood buffy coat samples from fifteen of twenty-two diabetics, all but four of whom were insulin-dependent and most aged under forty-five years. Intracutaneous injection of the same preparation into six diabetics with positive in vitro reactions induced typical delayed-type hypersensitivity in four, thus strengthening the validity of the results as an indication of in vivo cell-mediated immunity. These findings have subsequently been extended and confirmed by the same workers, using an antigen of homogenated fetal calf pancreas (Nerup et al, 1973a, 1974b) and by ourselves, using an antigen of homogenated human pancreas (MacCuish et al, 1974c; Figure 7). The lower proportion of positive results in these later studies is attributable in part to the low islet-cell content of pancreatic homogenates and in part to the inclusion of patients with insulin-independent types of diabetes.

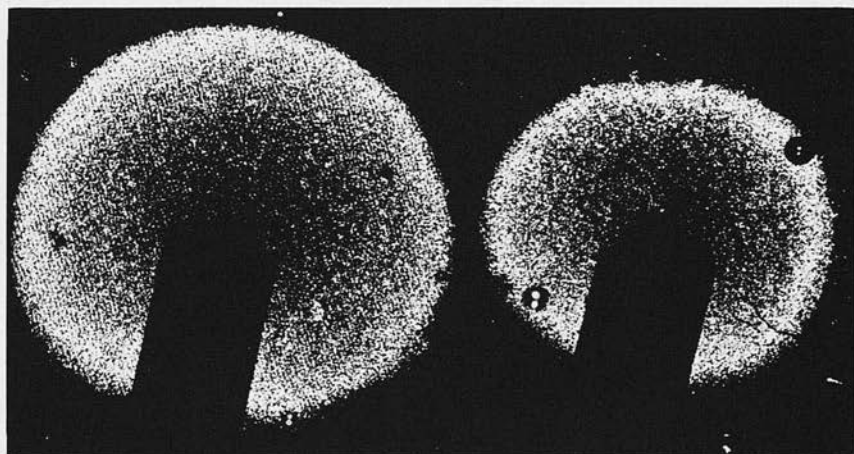


Figure 6. Inhibition of leucocyte migration in the presence of pancreatic antigen. On the left, leucocytes have migrated normally from the capillary tube into culture medium; on the right, migration has been inhibited by the addition of pancreatic antigen to the culture medium. Reproduced from MacCuish et al (1974c) by courtesy of the Editor of *Diabetes*.

The results of migration inhibition studies using pancreatic and other antigens in diabetes are summarised in Table 4. With respect to specific antigens, it now seems possible to state that considering the diabetic population as a whole, approximately thirty per cent of patients show evidence by the LMT of a state of cell-mediated immunity to an antigen which is present in the pancreas, is species-non-specific (demonstrable with porcine, bovine, human and rat pancreas), and is possibly different from insulin. The phenomenon is found in both juvenile-onset and maturity-onset diabetics, occurring in insulin-independent as well as insulin-dependent patients; it is, however, much more commonly found in young, recently diagnosed diabetics (irrespective of therapy) than in any other type of patient. It is of particular importance to note that antigen-induced migration inhibition has been

diabetes. Richens et al (1973) described striking migration inhibition of leucocytes from both young and elderly diabetics to mitochondrial antigens prepared from human and rat liver, and in a subsequent study (Richens et al, 1974) suggested that the antigenic component was localised to the inner mitochondrial membrane. This reactivity to liver mitochondria can of course be considered as a general nonspecific 'marker' of autoimmune disease, since inhibition of leucocyte migration is commonly induced by this antigen in patients with Hashimoto thyroiditis (Calder et al, 1972), pernicious anaemia (Goldstone et al, 1973) and primary biliary cirrhosis (Brostoff, 1970). Thus the findings of Richens et al, if confirmed, would provide further evidence for aligning diabetes mellitus with this group of diseases. However, it will be seen from Table 4 that the results obtained by Richens et al are as yet unconfirmed by other investigators using antigens prepared either from liver or from other mitochondrial-rich tissues (adrenal, kidney). The reasons for the differences between the various studies are not yet clear but in part may reflect differing methods of antigen preparation; until this question is resolved, it is not possible to make any definitive statement regarding the *in vitro* effects of mitochondrial antigens on diabetic leucocytes.

#### **Studies of lymphocyte transformation in response to insulin antigens**

The transformation of cultured small lymphocytes (presumably T cells) to lymphoblasts in the presence of antigen is another widely used *in vitro* test of cellular immune function. As with studies using a mitogen such as PHA, the effect of antigen is assessed by direct examination of cultured cell morphology using the light microscope or, more conveniently, by pulsing the cultures with a radioactive DNA precursor (e.g.,  $^3\text{H}$ -thymidine) which will be incorporated into the nuclei of transforming cells: the extent of blastogenesis can subsequently be measured by beta counting of the extracted nucleoprotein. In the context of autoimmune disease, the transformation technique has been used to demonstrate the presence of lymphocyte subpopulations sensitised to intrinsic factor in patients with pernicious anaemia (Tai and McGuigan, 1969) and to thyroglobulin in patients with Hashimoto thyroiditis (Ehrenfeld, Klein and Benezra, 1971). Human diabetes mellitus would seem to be a suitable model for the transformation technique in an attempt to demonstrate the existence of lymphocyte subpopulations which 'recognise' components or secretions of the endocrine pancreas. At present, however, there is little information concerning antigen-induced lymphocyte transformation in this disease although various investigators (Halpern, Ky and Amache, 1967; Federlin, Kreigbaum and Flad, 1968; Federlin, 1971) have shown that bovine insulin can induce blastogenesis in lymphocytes from diabetics with both immediate (urticarial) and delayed type (cutaneous) insulin allergy.

We have recently studied the effects of adding purified insulins and insulin chains to cultured lymphocytes from newly-diagnosed diabetics and insulin-taking patients without evidence of allergy (MacCuish et al, 1975). The extent of blastogenesis in these cultures was assessed both by morphological examination and by  $^3\text{H}$ -thymidine uptake. The results of some of these experiments are shown in Figures 5 and 6 and can be summarised as follows:

Table 4. *Studies of antigen-induced inhibition of leucocyte migration in diabetes mellitus, using various pancreatic and non-pancreatic antigens*

Investigators	Antigens used in the LMT		
	Positive results (inhibition)	Negative results (no inhibition)	Comments
Nerup et al (1971)	Porcine pancreas (islets)	Pig liver mitochondria, porcine kidney, porcine insulin, bovine insulin	Inhibition in 65% of 22 diabetics (most young, insulin-dependent)
Nerup et al (1973a, 1974a)	Fetal calf pancreas (homogenate)	Fetal calf liver, fetal calf thymus	Inhibition in 28% of 112 diabetics (all types)
Richens et al (1973)	Rat liver mitochondria, human liver mitochondria	Rat kidney mitochondria, rat adrenal mitochondria	Inhibition in 63% of 47 diabetics. Only study showing inhibition to mitochondria
MacCuish et al (1974)	Human pancreas (homogenate)	Rat liver mitochondria, human liver mitochondria, bovine insulin, bovine glucagon	Inhibition in 29% of 101 diabetics (all types)
Richens et al (1974)	Human pancreas (islet-cell tumour), rat pancreas (islet suspension)	—	Unpublished (personal communication)

LMT = leucocyte migration test.



3. Two-thirds of the patients whose cells had responded to intact insulin, also showed significant transformation when the lymphocytes were cultured in the presence of isolated bovine insulin B chain (Figure 9). In contrast there was virtually no blastogenic response when the cells were cultured in the presence of isolated bovine or porcine insulin A chain.

The blastogenic effect of intact insulin is perhaps not surprising in the group of established diabetics that were tested: all had been insulin-treated for months or years, virtually all would be expected to have humoral antibodies to injected insulin (Berson et al, 1956), and it is reasonable to suppose

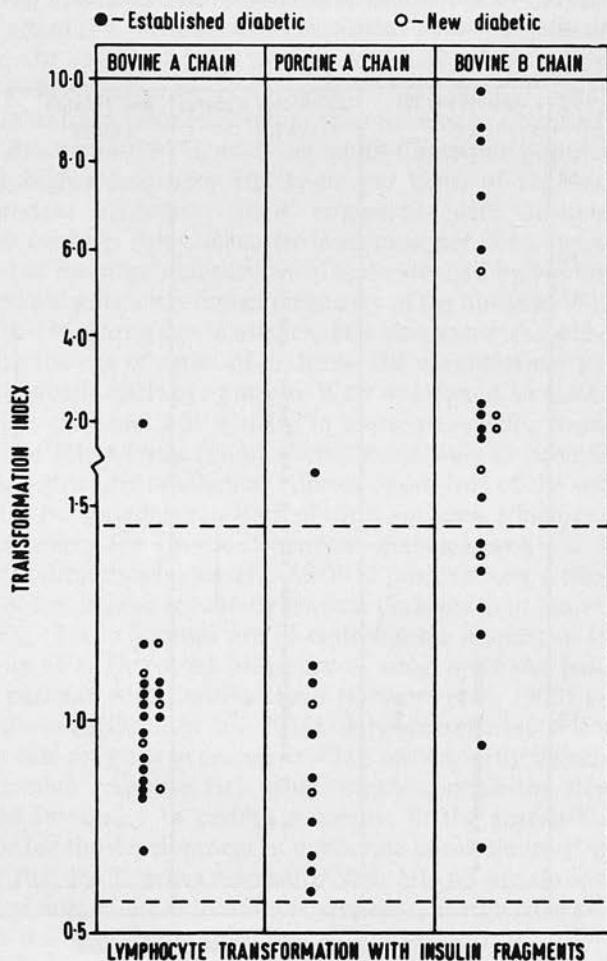


Figure 9. Lymphocyte transformation induced by bovine and porcine A chain and bovine B chain in 21 diabetics who responded to intact insulin. Symbols as in Figure 8. Closed circles = established diabetics; open circles = newly diagnosed diabetics. Significant transformation to either A chain is shown by only one diabetic, as compared to 14 (67 per cent) who respond to B chain. The effects of B chain differ significantly ( $P < 0.01$ ) from those of the A chain of either insulin. Reproduced from MacCuish et al (1975) by courtesy of the Editor of *Diabetes*.

1. Lymphocytes from a considerable proportion (approximately twenty-five per cent) of insulin-taking diabetics, who have no clinical sign of insulin allergy, undergo significant blastogenesis when cultured in the presence of insulin (Figure 8). Cells from diabetics which respond in this fashion to bovine insulin, which all the patients were receiving, will also respond in an identical fashion to porcine insulin.
2. Lymphocytes from five out of ten newly diagnosed diabetics also underwent significant transformation when exposed to bovine and porcine insulin (Figure 8). Two of these five patients had been insulin-treated for less than three weeks, while three had never received insulin or other antidiabetic therapy when their cells were studied.

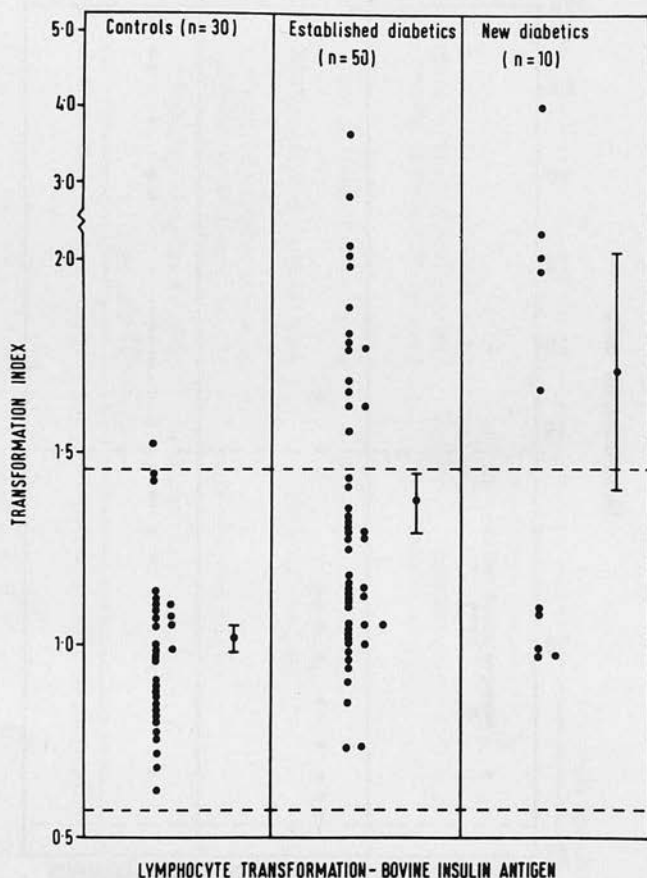


Figure 8. Lymphocyte transformation in controls and diabetics induced by bovine insulin antigen. The mean transformation index  $\pm$  s.e.m. is shown to the right of the individual results for each group. Dotted lines indicate the normal range (mean  $\pm$  2 s.d.) of transformation indices in control subjects. Significant transformation (index  $>$  1.45) is shown by 15 established and 5 newly diagnosed diabetics as compared to one control. Both diabetic groups differ significantly ( $P < 0.01$ ) from the control group. Reproduced from MacCuish et al (1975) by courtesy of the Editor of *Diabetes*.

comparable to that demonstrated in autoimmune thyroid disease (Calder et al, 1973a and b). Again, however, diabetes is a suitable disease model for the application of these techniques and it may be anticipated that they will soon be applied in this field.

### **HISTOCOMPATIBILITY (HL-A) ANTIGEN TYPING IN DIABETES MELLITUS**

There is disagreement regarding the results of histocompatibility (HL-A) antigen typing in diabetics. Finkelstein, Zeller and Walford (1972) found no significant alteration in HL-A antigens in forty-four insulin-dependent patients but the validity of this study is dubious as the patient population was racially heterogeneous (Caucasian, Mexican and Negro) and any alterations in antigen frequency might thus have been obscured. In contrast, Singal and Blachman (1973), studying an all-Caucasian population, found a significantly higher frequency (thirty-six per cent) of the antigen W15 in insulin-dependent diabetics when compared with insulin-independent patients and controls (prevalence ten and nine per cent, respectively). The latter study has recently been confirmed and extended by Nerup et al (1974b) who reported a significantly higher frequency of the antigens W15 and HL-A8 in a series of 146 Caucasian diabetics, and correlated the presence of these antigens with the age of onset of diabetes, the weight of the patient and the type of antidiabetic therapy: antigen W15 was found in both juvenile and maturity-onset patients, but usually in association with insulin-dependent diabetes, while HL-A8 was found almost exclusively in juvenile-onset, non-obese, insulin-dependent diabetics. Almost two-thirds of the subjects studied by Nerup et al possessed one or both of these antigens, which can be regarded as genetic markers for insulin-dependent diabetes, and the authors have estimated that subjects who are HL-A8/W15 positive have a two to three-fold higher risk of developing insulin-dependent diabetes than individuals lacking these antigens. These findings are of considerable interest in the context of autoimmunity as an increased frequency of antigen HL-A8 has already been detected in patients with thyrotoxicosis (Grumet et al, 1973) and idiopathic Addison's disease (Platz et al, 1974). Histocompatibility complexes like HL-A carry not only the genes controlling serologically detectable antigens but also immune response (Ir) genes which control the development of cell-mediated immunity to certain antigens; in the search for a common denominator for the development of endocrine autoimmunity, it is intriguing to speculate that the Ir genes associated with HL-A8 are those which permit the full expression of an autoimmune response, irrespective of the stimulus which elicits this response.

### **PATHOLOGY OF THE PANCREAS IN DIABETES MELLITUS**

A number of pathological studies over the past decade have described the morphological findings in the pancreata of various clinical types of diabetes mellitus; the major conclusions are summarised in Table 5. The appearance of the pancreas in acute juvenile-onset patients is naturally of especial

that some have developed cellular immunity in the form of an insulin-sensitised lymphocyte subpopulation. Many mammalian cells have receptors for insulin and this has also been shown to be true for human lymphocytes (Gavin et al, 1973). The phenomenon appears to be relatively common in diabetics who have no clinical sign of insulin allergy, and may be regarded as a sensitive *in vitro* measurement of an insulin hypersensitivity that is not of clinical significance.

More interesting is the finding of insulin-induced lymphocyte transformation in a small number of newly-diagnosed patients who were either untreated or insulin-treated for a very brief period. Further studies will be required to confirm these observations in larger numbers of untreated diabetics but the initial results at least allow the tentative suggestion to be made that a proportion of such patients have an insulin-sensitised lymphocyte population before exogenous insulin has been given and before humoral insulin antibodies have developed. Thus the existence of this cell population may reflect a state of cell-mediated autoimmunity, to insulin or insulin precursor, in early diabetes, and may be the analogue to the population detected in these patients by the LMT.

Two conclusions are possible from the studies of lymphocyte transformation induced by isolated insulin chains (Figure 6). In the first place, the striking blastogenic effect of isolated B chain (which is metabolically inert) strongly implies that the blastogenesis induced by intact insulin molecule is a true immunological effect, rather than some metabolic effect brought about by a hormonal action of insulin on the insulin receptors of lymphocytes. Secondly, the clearcut difference between the effects of the two major polypeptide chains (A chain being inactive, B chain highly active) might suggest that the B chain is the major antigenic site determining cellular hypersensitivity to insulin. The role of B chain in this context may be contrasted with the humoral response to exogenous insulin, where various studies suggest that the A chain is the major determinant of antibody production (Berson and Yalow, 1959; Wilson, Dixon and Wardlaw, 1962). Thus it is possible that differing antigenic activity, whether cellular or humoral, resides at different sites on the insulin molecule, although the factors governing the expression of such activity remain obscure. However, it must also be remembered that an apparent lack of cellular antigenicity of A chain might be explained by the physical changes undergone by this peptide during the splitting of insulin; isolated A chain is 'stretched' by comparison with its configuration in the intact insulin molecule and the intra-chain disulphide bridge between 6 and 11 is broken, with the formation of cysteic acid residues which could prevent antigen-antibody interaction at this site. It is to be hoped that further studies of antigen-induced lymphocyte transformation may delineate more clearly the cellular antigenic portion of insulin and may indicate whether related proteins or precursors (e.g., proinsulin, C-peptide) have similar properties in early diabetes.

#### **Other tests of cell-mediated immunity in diabetes mellitus**

As yet there is no published information on lymphocyte cytotoxicity or on lymphocyte-dependent antibody-mediated (K-cell) cytotoxicity in diabetics



relevance to the aetiology of diabetes mellitus, and in this connection the finding of 'insulitis' has excited particular speculation. The term 'insulitis' was first coined by von Meyenburg (1940) to describe the inflammatory lesions in the islets of Langerhans of some diabetics, although such lesions had in fact been described by the early investigators of diabetic pathology at the beginning of this century, well before the era of insulin treatment (Schmidt, 1902; Heiberg, 1911). The lesions of insulitis may be heavy, involving the majority of the islets, or light, involving only scattered islets. Affected islets are usually of the atrophic type and infiltrated with lymphocytes, commonly round their periphery but sometimes throughout the islet. Large mononuclear cells, sometimes polymorphs and very rarely eosinophils may also be present in the infiltrate, but plasma cells have never been observed. The most common appearance seems to be of a 'halo' of lymphocytes just outside the capsule of the islet, extending a little way into the periphery of the endocrine tissue and probably cuffing capillaries in the islet stroma (Warren, LeCompte and Legg, 1966); more diffuse lymphocytic infiltration, overrunning the whole islet, is less commonly encountered (Gepts, 1965). The lesion in juvenile diabetes is quite distinct from the insulitis which occurs in some infants of diabetic mothers; the latter condition is characterised by an infiltrate which is predominantly of polymorphs, includes many eosinophils, and is without effect on pancreatic insulin production.

For many years insulitis was considered to be a very rare phenomenon, confined to a few cases of diabetes of recent onset in children (Warren and Root, 1925; LeCompte, 1958). It has been only briefly mentioned, or not at all, in many textbooks on pathology or diabetes and many pathologists have never seen a case (LeCompte and Legg, 1972). The finding by Gepts (1965) of insulitis in fifteen out of twenty-two young diabetics, i.e., more than two-thirds of the pancreata that he examined, was therefore in direct contrast to this classical opinion and several reasons can be advanced which suggest that the rarity of this lesion is more apparent than real. First, comparatively few pathologists now have the opportunity to examine by modern techniques the pancreas of juvenile-onset diabetes shortly after the onset of disease, and this is of course a direct result of the advent of insulin therapy. Secondly, it has never been detected in young diabetics who have died more than twelve months after the onset of disease, by which time the changes in the pancreas are so extensive (Table 5) as to obscure any subtle cellular infiltrate. Thirdly, it has never (with one exception) been described in maturity-onset diabetes, the exception being the cases detected by LeCompte and Legg (1972), who found insulitis in two elderly diabetics with brittle, atypical maturity-onset diabetes that required insulin treatment in each case shortly before death. Fourthly, insulitis may in any case be a transient phenomenon which is present only for months or even weeks during the development of clinical diabetes. Finally, the lesion may be so slight as to be easily overlooked, affecting only a few islets and perhaps detectable only with the electron microscope. Against all these plausible arguments must however be set the recent findings of Doniach and Morgan (1973) who were unable to find any insulitis in the pancreata of nine juvenile-onset patients who died within a few weeks of diagnosis. A balanced interpretation of all the present literature

Table 5. *Pathology of the pancreas in various clinical types of diabetes mellitus*

References	Morphological feature	Types of diabetes mellitus studied		
		Acute, juvenile-onset <sup>a</sup>	Chronic, juvenile-onset <sup>b</sup>	Maturity-onset <sup>c</sup>
Ogilvie (1964)	Number of islets	Reduced	Markedly reduced	Moderately reduced
	Hypertrophy of remaining islets	Present, not invariable	Absent	Absent
	Atrophy of remaining islets	Rare	Gross	Seldom seen
Gepts (1965)	Cell numbers	Marked reduction	Virtually absent	Moderate reduction
Warren, LeCompte and Legg (1966)	Cell activity	Some hyperactive	Majority inactive	Majority normal
Doniach and Morgan (1974)	'Insulinitis'	Present in over 50% of cases <sup>d</sup>	Absent	Absent
	Weight of pancreas	Usually normal	Markedly/moderately reduced	Usually normal
	Exocrine pancreas	Focal acute pancreatitis	Diffuse fibrotic pancreatitis	Diffuse pancreatic fibrosis

<sup>a</sup>Onset under age 22, most untreated at time of death. Death within year of diagnosis (most within 6 months).

<sup>b</sup>Onset under age 30 (most under age 22). Insulin-treated for at least 2 years (average 18 years).

<sup>c</sup>Onset over age 40. Insulin-independent.

<sup>d</sup>Not observed by Doniach and Morgan.

heterologous anti-insulin serum) and active immunisation procedures (i.e., injection of homologous or heterologous insulin) have been successful in producing insulinitis in a variety of species, and in the context of autoimmunity the models described by Renold and his colleagues and by Nerup et al (1973b) are of particular interest. These investigators have shown that the injection of homologous insulin in cattle or sheep, or a suspension of homologous endocrine pancreas in rodents can be followed by pathological changes in the pancreatic islets which strongly resembles the lesions of insulinitis found by Gepts (1965) in human diabetes. Insulinitis in these experimental animals does not always lead to the development of overt diabetes mellitus but is accompanied by the production of humoral anti-insulin antibodies (Renold, Soeldner and Steinke, 1964) or of antipancreatic cell-mediated hypersensitivity (Nerup et al, 1973b). The findings have obvious implications for the hypothesis of autoimmune diabetes in man, although it has not yet been possible in any animal model to fully characterise the antigen(s) responsible for the development of the insulinitis lesion and the appearance of sensitised lymphocyte subpopulations.

#### VIRAL INFECTION AND AUTOIMMUNITY IN DIABETES MELLITUS

The suggestion that viral infection might be aetiologically related to diabetes mellitus was apparently first offered by Gundersen (1927), based on his observation that an attack of mumps might be closely followed by the development of clinical diabetes. Since then it has been established both clinically and experimentally that viral infection can invade the pancreas of many animal species. The induction of carbohydrate intolerance by viral inoculation of the experimental animal was first accomplished by Craighead and McLane (1968), who inoculated mice with the M variant of the encephalomyocarditis (EMC) virus to produce a disease which clinically and pathologically resembled human, abrupt-onset diabetes with insulinitis. Numerous subsequent investigators have since documented the ability of variants of the EMC virus and of group B Cocksackie viruses to induce a diabetes-like syndrome in rodents (Craighead and Steinke, 1971; Muntefering, Schmidt and Korber, 1971; Wellman et al, 1972; Coleman, Gamble and Taylor, 1973). From these and other studies it has become clear that differing strains or variants of these viruses can cause either a diffuse pancreatitis or more specific lesions of the endocrine pancreas. Thus the inoculation of the E variant of EMC virus and Cocksackie B3 virus induces a generalised pancreatitis without specific insular damage or the development of diabetes, while the M variant of EMC virus and Cocksackie B4 virus produces only a mild exocrine pancreatitis but a severe, rapidly-evolving insulinitis which is invariably accompanied by diabetes. These latter 'specific' insulinitis-producing viruses seem to produce a typical series of pancreatic changes: within forty-eight hours of their inoculation many islets show interstitial oedema and marginal or central necrosis of cells. On the third and fourth days, there is extensive and total necrosis of some islets, both  $\alpha$  and  $\beta$  cells being involved, and macrophages begin to infiltrate between the islet-cells at this time.

might therefore be summarised as indicating that insulinitis is not a particularly rare lesion, but nor is it as common as the findings of Gepts would suggest, and overall it probably occurs at some time in something under half the cases of juvenile diabetes mellitus.

### The production of insulinitis in experimental models

Several theories have been advanced as to the cause of insulinitis (and per se as to the possible cause of onset of diabetes). Early workers suggested that the lesion might represent destruction of the islet cells either by over-stimulation or by some unidentified cytotoxic agent (von Meyenburg, 1940). Both explanations seem unlikely as no other similar lesions have ever been observed in other endocrine glands submitted to overstimulation, nor do they appear in the islets of alloxan-induced diabetes. The two present theories which have received the strongest support from clinical and experimental studies are the viral and immunological hypotheses respectively. Epidemiological and other evidence for viral infection as a cause of insulinitis and diabetes is considered below. With respect to the immunological hypothesis, the various animal models in which insulinitis has been induced by immune mechanisms are summarised in Table 6. It will be seen that both passive (i.e. injection, of

**Table 6.** *Summary of the pathological findings in various animal species following the induction of insulinitis by passive and active immunisation*

Method	Species	Reference	Findings
Injection of heterologous (guinea-pig) anti-insulin serum	Rat	Lacy and Wright (1965)	Diffuse exocrine pancreatitis, acute transient insulinitis (mainly eosinophils), carbohydrate intolerance Chronic insulinitis (mainly monocytes)
	Mouse	Logothetopoulos and Bell (1968)	
	Mouse	Freytag (1972)	
Injection of heterologous insulin (in CFA)	Cattle	Renold, Soeldner and Steinke (1964)	Chronic insulinitis (mononuclear), fibrosis of some islets, exocrine pancreas normal, humoral anti-insulin antibody production, diabetes in a few animals
		LeCompte et al (1966)	
	Sheep	Federlin, Renold and Pfeiffer (1968)	
	Rabbit	Toreson et al (1964) Grodsky et al (1966) Lee et al (1969) Kloppel, Altenahr and Freytag (1972)	
Injection of homologous insulin (in CFA)	Cattle	Renold, Soeldner and Steinke (1964)	Identical to experiments using heterologous insulin
	Sheep	LeCompte et al (1966) Federlin, Renold and Pfeiffer (1968)	
Injection of homologous and heterologous endocrine pancreas (in CFA)	Rat	Nerup et al (1973a) Andersen et al (1974)	Insulinitis (mononuclear), $\beta$ -cell degeneration, exocrine pancreas normal, antipancreatic cellular hypersensitivity (by the LMT), transient diabetic state

CFA = Complete Freund's adjuvant.



host. These mechanisms would provide at least the theoretical basis for a relationship between virus infection and human diabetes with insulinitis, although this relationship still remains to be proved. On the one hand it is encouraging to note that Craighead (1974), examining histological material from infants dying with fully-documented Coxsackie B virus infections, has found changes in the pancreata (fibrosis, interstitial pancreatitis, lymphocytic infiltration of the islets of Langerhans) identical to those described by Gepts (1965) in his cases of juvenile diabetes with insulinitis. On the other hand, it is salutary to record that to the date of this review, no published work has yet described the culture of any virus from the blood, pancreatic tissue or excreta of a newly-diagnosed diabetic patient.

### IMMUNOLOGICAL ABNORMALITIES IN RELATION TO THE COMPLICATIONS OF DIABETES MELLITUS

Microangiopathy is the most serious specific complication of longstanding diabetes mellitus and the general features of this lesion (basement membrane thickening, deposition of certain plasma proteins, hyalinisation and loss of function in small blood vessels) are such as to arouse speculation that they may be due to abnormal immunological reactions. It should be said at the outset that at present there is no evidence of any kind to suggest that microangiopathy is related to autoimmune disease mechanisms in diabetes, and research in this area remains a major future endeavour. However, the remarkable failure of pathologists to recognise a specific diabetic microangiopathy prior to the insulin era (Kimmelstiel and Wilson, 1936) and the detection of insulin antibodies in virtually all insulin-treated diabetics, prompted Berson et al (1956) to suggest that the vascular lesion in diabetes was the result of insulin therapy and was caused by deposition of insulin-antibody complexes on the basement membrane of the renal and other microvessels.

There is a certain amount of experimental evidence to support this immune complex hypothesis: renal tissue from diabetics shows fine granular deposits on the basement membrane, resembling those found in post-streptococcal and membranous glomerulonephritis (Bloodworth, 1968), while the glomeruli in diabetic nephropathy have insulin-binding capacity and also contain immunologically-detectable insulin (Berns et al, 1962). The latter features are also seen in the vessels of the diabetic eye (Coleman et al, 1962). The blood of some diabetics is said to contain insulin antigen-antibody complexes (Jayarao et al, 1974) and perhaps even more interesting is the recent observation that insulin and human IgG can react *in vitro* under mild reducing conditions to produce a complex that has high potency for complement fixation (Cantrell, Stroud and Pruitt, 1972). The latter mechanism would be considered an immunologic 'bypass' since specific antibody to insulin is not required.

On the other hand, the evidence against some form of immunity to insulin as the basis for diabetic angiopathy is rather more convincing (Editorial, 1972). Immunohistopathological studies have revealed that the basement membrane in diabetic glomerulosclerosis contains not only insulin, IgG,

Between the third and the fifteenth day, but most commonly after about a week, poorly developed inflammatory infiltrates of the pancreas are seen, and the islets are infiltrated by mononuclear round cells and a few polymorphs. Marked degeneration of the surviving  $\beta$  cells is evident. The lesions are always patchy, many islets being initially normal on light microscopy, but latterly these ostensibly unharmed islets exhibit advancing  $\beta$ -cell degranulation. The extent of islet-cell damage seems to be partly dependent on the metabolic state of the animal at the time of inoculation; mice who have been pretreated with steroids or rendered obese by gold-thio-glucose injection show particularly severe lesions (Craighead, 1966), suggesting that  $\beta$  cells which are most active metabolically are also the most vulnerable to damage by viral invasion.

Viral diseases which are known to invade the pancreas have recently been summarised by Steinke and Taylor (1974); in man, these include infection with the mumps, rubella and infectious mononucleosis viruses as well as the group B Cocksackie viruses. A possible clinical significance for these observations in relation to the development of human diabetes was proposed by Gamble and Taylor (1969), who initially described an increased incidence of cases of diabetes in the autumn and winter seasons, and subsequently correlated these cases with the seasonal prevalence data for Cocksackie type B4 virus infections. By retrospective serological testing they were able to show that the sera of insulin-dependent diabetics of less than three months' duration contained a significantly higher antibody titre to Cocksackie B4 than the sera of non-diabetic normal controls or longstanding diabetic patients (Gamble et al, 1969). These observations are interesting but require confirmation, preferably on a prospective basis, and it is curious that later studies by the same authors (Gamble, Taylor and Cumming, 1973) and others (Baum, Aynsley-Green and MacCallum, 1974) have apparently failed to show any association between viral infection and the development of diabetes in children under the age of twelve years.

The possible mechanisms whereby viral infection of the pancreas or other tissue could initiate immunopathological processes have been well reviewed by Freytag (1974). Virus invasion of the host is followed by two differing cell changes according to the general properties of viruses: the virus either multiplies and matures in one cell and after cytolysis invades other cells, producing spreading infection, or there is a steady-state interaction in which the virus replicates in the host cells, leaving the host structurally intact but producing functional alterations within the infected cells. Either or both of these virally-mediated cell changes can lead to a wide variety of different immunological reactions which probably all have cytotoxic effects. In the specific context of autoimmunity, Freytag (1974) concludes that viral infections can give rise to:

1. New antigenic sites in the protein of cells.
2. The production of cell-specific antibodies and sensitised lymphocytes.
3. A nonspecific acceleration of general immune responses.

By such cellular changes, virus infection may trigger off an autonomous development of progressive chronic inflammatory processes, i.e., autoimmune processes, even though the virus itself has already been overcome by the

in organ-specific autoimmune disease (adrenal, thyroid, stomach) and the autoimmune analogy is considerably strengthened by the recent findings, described in this chapter, of sensitised lymphocyte populations and circulating islet-cell antibodies in human diabetes. It is, however, unlikely that autoimmunity could be the sole cause of the insulinitis lesion as islet-cell antibodies seem to be a rare finding in diabetes, except in patients with coexistent overt autoimmune disease, and insulinitis itself is not accompanied by the plasma-cell infiltration and lymphoid-follicle formation that are regarded as classical findings in the 'accepted' organ-specific autoimmune diseases. Infection with group B Coxsackie or similar viruses has also been postulated as a cause of insulinitis and it is important to remember that the autoimmune and the viral hypotheses are not mutually exclusive, i.e., that an autoimmune process might well be triggered off by viral infection. It is tempting to speculate that the nature and duration of the immune response to viral infection is primarily determined by the immune response gene(s) of the infected patient, which in turn is probably dependent on HL-A type. One might then construct a unifying hypothesis whereby young subjects, having inherited a particular type of immune response gene by virtue of their HL-A type, respond to (say) a viral infection by initiating an irreversible and relentless series of autoimmune processes which culminate in destruction of the endocrine pancreas and the development of clinical diabetes (Editorial, 1974).

The studies reviewed in this chapter lend strong credibility to the concept of a true autoimmune form of diabetes mellitus, which has now been shown to fulfil most of the classical criteria (close relationship with other autoimmune diseases, lymphocytic infiltration of the end-organ, circulating humoral antibody directed against the end-organ, circulating lymphocytes sensitised against the end-organ). It is not yet possible to suggest what proportion of patients with diabetes have developed their disorder on the basis of autoimmunity but the available evidence indicates that this syndrome will be found most frequently in young insulin-dependent diabetics, predominantly of female sex, who possess a variable pattern of circulating autoantibodies, a specific pattern of HL-A complexes and may also have clinical or laboratory evidence of recent viral infection. It may be anticipated that future studies of autoimmune diabetes will explore the interrelationships between these various factors, examine immune mechanisms in such patients by a variety of sophisticated techniques, and examine the possibility (as yet without foundation) that autoimmunity may have some significance for the development of diabetic microangiopathy.

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IgM and B<sub>1</sub>C fraction of complement, but also contains 'non-immunological' plasma proteins (albumin, caeruloplasmin, etc.) deposited in linear fashion (Westberg and Michael, 1972). A similar miscellany of plasma proteins and lipoproteins, as distinct from pure immunoglobulins, has been found in the microvessels of skin biopsies from diabetics (Larsson, 1967). These studies provide powerful grounds to argue that immune mechanisms are not primarily responsible for the deposition of immunoglobulins and other proteins in diabetic basement membrane, and that the abnormalities are more in keeping with a nonspecific trapping (binding) of serum proteins. Further evidence against a pathogenic role for immune complexes in this context is given by the demonstration that linear deposits of IgG in diabetic kidneys are unable to fix complement (Westberg and Michael, 1972), which suggests strongly that the IgG is not bound to antigen. Chemical analysis of diabetic glomeruli shows an increase in both the protein and the carbohydrate components, again suggesting metabolic rather than immunologic disturbance (Churg and Dolger, 1971), and perhaps the strongest argument against the insulin-immunity hypothesis is provided by the findings that basement membrane thickening may precede carbohydrate intolerance in diabetes (Siperstein et al, 1968) and that classical diabetic microangiopathy may occur in patients who have never received insulin (Rifkin, Leiter and Berkman, 1941) or even in persons with normal glucose tolerance (Daysog, Dobson and Brennan, 1961). The hypothesis of insulin immune complexes as the cause of diabetic microangiopathy is thus probably untenable. Further speculation as to the cause of the lesion is beyond the scope of this chapter but current suggestions include a fundamental disturbance of carbohydrate metabolism (Churg and Dolger, 1971), an inherited genetic defect associated with diabetes and occurring with or before carbohydrate intolerance (Siperstein, 1970) and a primary defect of the mesangial cell in diabetes (Kimmelstiel, 1968). These suggestions are by no means mutually exclusive but all point to metabolic rather than immunological abnormality.

## SUMMARY AND CONCLUSIONS

An increasing weight of indirect and direct evidence suggests that autoimmune mechanisms have an important role in the aetiology of some cases of human diabetes mellitus. With regard to the indirect evidence, diabetes is commonly associated with overt or subclinical autoimmune disease and may appear as part of a polyendocrine autoimmune syndrome. Organ-specific autoantibodies are frequently found in diabetic sera, directed especially against antigens in the thyroid or stomach, and are present to excess in the relatives of these diabetics. Juvenile-onset insulin-dependent diabetics often possess the same histocompatibility (HL-A) antigens that are found in patients with thyroid or adrenal autoimmune disease, and these HL-A complexes carry not only the genes of serologically detectable antigens but also immune response genes which may permit the full expression of an autoimmune response.

With regard to the direct evidence, insulinitis is a relatively common finding in the pancreata of newly diagnosed juvenile-onset diabetics. Insulinitis has inevitably been compared with the very similar round-cell infiltrates found



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